iGEM CDI Project Team

Lab Journal

Researcher Initials Key

AC = Alan Chen
AG = Amy Guo
AP = Amy Pai
JL = Jared Lamar
OE = Omar Ebrahim

June 27th

AC/OE

Inoculation of strains obtained from Dr. Christopher Hayes at UC Santa Barbara

- 10mL LB; 37C for 16h shaking
    - EPI100 w/ EC869 cosmid
        - 10uL ampicillin
    - X90 w/ EC93 cosmid
        - 10uL ampicillin
    - *Enterobacter aerogenes* (ATCC 13048)

June 28th

AC/OE

Mini-prep of EC93 and EC869 containing cosmids (x2 each)

-ZR Plasmid Miniprep Kit from Zymo

- EC869
    - 145.42 ng/µL
    - 198.85 ng/µL
- EC93
    - 90.81 ng/µL
    - 45.95 ng/µL

Isolation of *Enterobacter aerogenes gDNA*

-Using QIAGEN Progene Yeast/Bacteria KitB

    - 34.87 ng/µL
    - 1.85 260/280

- Made glycerol stocks (500uL culture, 500uL 50% glycerol)
- Gibson primers designed for CDI assemblies in pSC101 AND pSB1C3

June 29th

- 12 new primers ordered from IDT
    - 4 for ATCC 13048 in pSB1C3
        - retain endogenous promoter instead of T7 promoter since membrane proteins are detrimental when overexpressed
    - 8 for Aerogenes pSC101

        - pSC101 backbone replacement for ColE1 (too high copy)

AC

- Amplified Gibson EBL2 fragments 2 (Second Half of CDI-A) and 3 (CDI accessory factors)
    - Diluted stock primer solution 10 fold (100µM to 10µM)
    - Diluted gDNA to 1ng/µL
        - Original was 35.8ng/µL
        - Added 34µL water to 1µL of stock gDNA
    - Used Q5 2x protocol for 50µL reaction
        - 2.5µL forward and reverse primers
        - 1µL of gDNA as template
        - 25µL Q5 2x Master Mix
        - 19µL water
    - PCR Cycle Protocols
        - 98C----->30s
            - Following done for 28 cycles
            - 98C----->10s
            - 66C----->30s
            - 72C----->140s
        - 72C----->2min
        - 14C----->Infinite

June 30th

- Yesterday's PCR failed and showed no band other than primer dimers
    - Could be issue with gDNA concentration (we assumed 1ng would be enough)
        - Troubleshoot with increased gDNA concentrations
    - Also test with different polymerase, use KapaHiFi instead

Troubleshoot: <u>PCR EBL2 Gibson frag 2 & 3 w/ varying gDNA template amount</u>

4 PCR Rxns: (amplifying 3kb frag 3)

- Q5 2x protocol for 25µL reaction
    - 1.25µL forward and reverse primers
    - 1) 1 ng 2) 10 ng 3) 34.87 ng 4) 69.74 ng template gDNA
    - 12.5µL Q5 2x Master Mix
    - 9µL water
- PCR Cycle Protocols
    - 98C----->30s
    - 28 cycles
        - 98C----->10s
        - 65C----->30s
        - 72C----->88s
    - 72C----->2min
    - 14C----->Infinite

Expecting: 3 kb Fragment

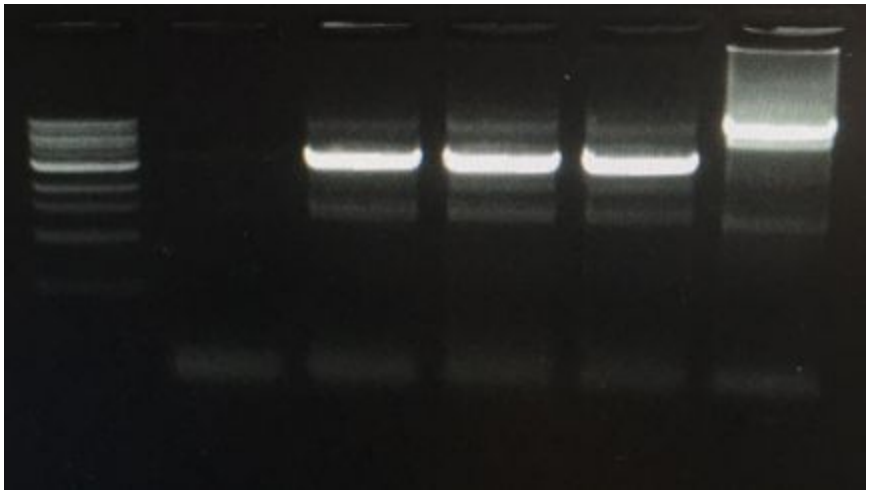
<u>Run separate rxn using KapaHiFi instead of Q5 in a 25µL rxn</u>

- KapaHiFi protocol:
    - 12.5µL KapaHiFi Master mix
    - 0.75µL forward and reverse primers
    - ~10ng of gDNA
    - Fill with water until reach 25µL
    - PCR Cycle Protocols (Amplifying 7.8kb frag 2)
        - 98C----->30s
        - 28 cycles
            - 98C----->10s

- 65C----->30s
- 72C----->2min 40s

- 72C----->2min
- 14C----->Infinite

Expecting: 7.8kb fragment



Ladder 2ng 10ng 34.87ng 69.74ng KapaHiFi

July 1st

- ordered CPCR and Gibson primers for toxin switch of Enterobacter aerogenes CDI loci 1 and 2

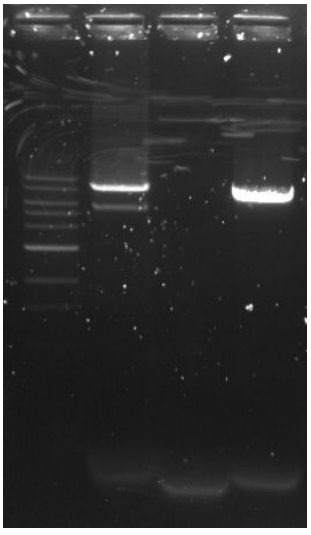
AC

PCR Protocol

10ng of gDNA
12.5uL of Q5 Master Mix
1.25uL of forward and reverse primers
Fill with water up to 25uL

Run 25 uL reaction

Loci 2 Fragment 2: 65C (3 min 54s extension time on 28x cycle)
Loci 1 Fragment 2: 66C (3min 30s extension time on 28x cycle) Fragment 3:64C(2min 30 extension time on 28x cycle)
Everything else was the same as the standard Q5 protocol



Ladder/ EBL2 frag 3/ EBL1 frag 2/ EBL1 frag 3

AG

- gel purified all EBL2 frag 3 bands (x3), EBL2 frag 2, and EBL1 frag 3

Nanodrop

EBL2 frag 3

10ng: 66.99ng/uL ; 1.84

34ng: 65.85ng/uL ; 1.89

68ng: 59.41ng/uL ; 1.98

EBL1 frag 3

43.40ng/uL; 2.03

EBL2 frag 2

19.13; 1.86

July 5th

AC

EBL1 frag 2:

Redoing failed PCR, modified protocol by having elongation time be extended to be 30s/kb, annealing temperature set to 72C, ran 2

rxns (25uL and 50uL)

Using standard Q5 protocol for 25uL

Using standard Q5 protocol for 50uL

- 98C----->30s
- Repeat with 28 cycles
    - 98C----->10s
    - 66C----->30s
    - 72C----->3min 30s
- 72C----->2min
- 14C----->Infinite

EBL2 frag 4:

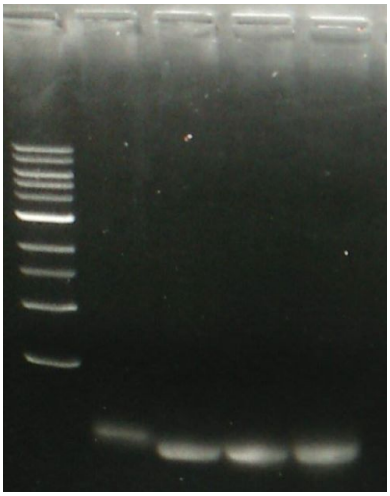
Using standard Q5 protocol for 25uL

- 98C----->30s
- Repeat with 28 cycles
    - 98C----->10s
    - 64C----->30s
    - 72C----->2min
- 72C------>14C

EBL2 frag 1:

Using standard Q5 protocol for 25uL

- 98C----->30s
- Repeat with 28 cycles
    - 98C----->10s
    - 65C----->30s
    - 72C----->3min
- 72C------>14C

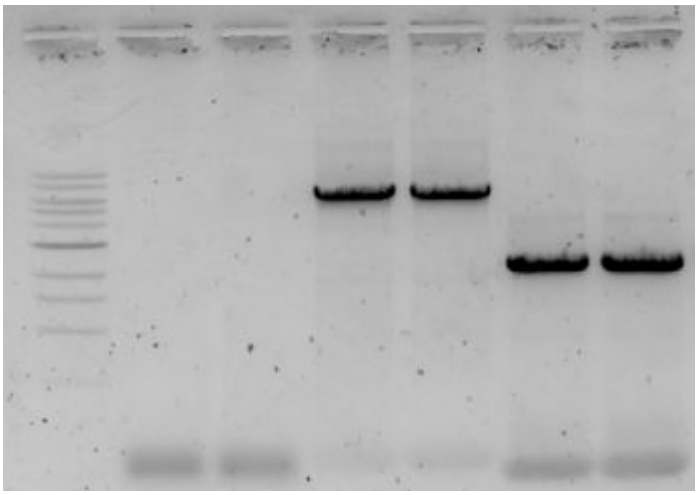


ladder/ EBL2 frag 1/ EBL2 frag 4/ EBL1 frag 2 (25uL rxn)/ EBL1 frag 2 (50uL rxn)

July 6th

AC/OE

- PCR fragments that didn't work
    - EBL2
        - Fragment 4 (pSB1C3), fragment 1 (gDNA)
    - EBL1
        - Fragment 2
- Changes to PCR
    - Performed two 25uL reactions (doubled and quadrupled original template DNA amount)
        - 20ng gDNA/40ng gDNA
        - 2ng pSB1C3/4ng pSB1C3
    - Instead of original 28 cycles, did 32 cycles of PCR
        - Other times and temperatures of individual steps remained the same as yesterday



Ladder/ 2x EBL1 frag 2/ 4x EBL1 frag 2/ 2x EBL2 frag 1/ 4x EBL2 frag 1/2x EBL2 frag 4/4x EBL2 frag 4

- EBL1 fragment 2 failed again -----> most likely faulty primers
- EBL2 frag 2 and 4 worked by increasing template DNA
- Eluted all fragments into 10uL of elution buffer
    - 2x EBL2 frag 4
        - 17.51 ng/uL
    - 4x EBL2 frag 4
        - 21.49 ng/uL
        - 22.83 ng/uL

- 2x EBL2 frag 1
  - 24.94 ng/uL
  - 20.63 ng/uL
- 4x EBL2 frag 1
  - 14.51 ng/uL

July 7th

AG/OE

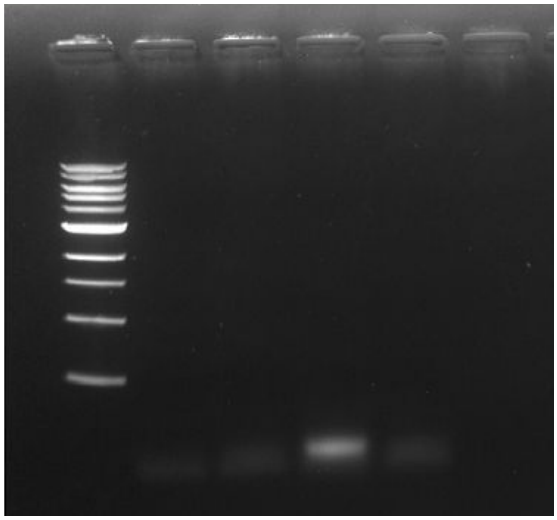
1. transform Kan^R into DH5-alpha (target) and transform EC93 cosmid into DH5-alpha (inhibitor)

July 8th

AG

PCR of EBL1 frag 2

- remade 10uM primer dilutions using same stock of 2F and 2R
- tried 10 and 20 ng amt of template DNA using both old and new aliquots
- 94 C for 30s
- 32 CYCLES:
  - 94 C for 10 s
  - 66C for 30 s
  - 72 C for 3:30 min
- 70 C for 20 s (final ext)
- 14C infinite hold



ladder/ old 10ng/ old 20ng/ new 10ng/ new 20ng

- observed growth on both 1:1 and 1:100 plates for DH5-alpha transformed w pSK33/EC93 cosmid
  - make 10uL starter cultures

July 9th

AG

- miniprep 5mL of each 10 mL starter culture
  - pSK33 insufficient yield
- test digest of EC93 with Hayes' strain as a control

<u>X90 w/ EC93</u>

- 500 ng DNA (14uL)
- 5uL NEB 2.1
- 1 uL EcoRI
- H2O to 50uL

DH5-alpha EC93
- 500 ng DNA (10.1uL)
- 5uL NEB 2.1
- 1 uL EcoRI
- H2O to 50uL

July 12th

- ordered: EBL1, EBL2 toxin switch CPCR and Gibson primers

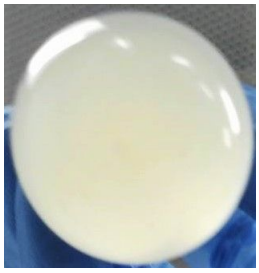
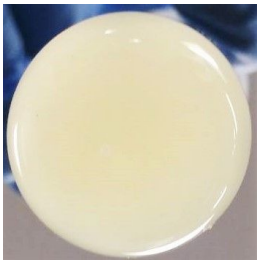
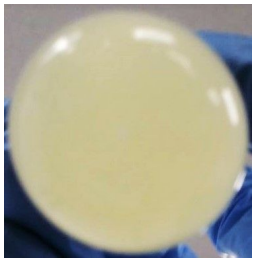
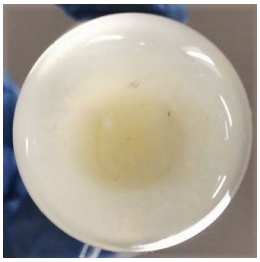
AG/OE

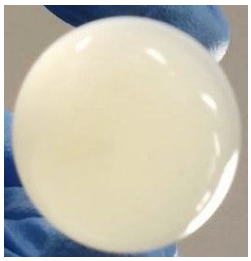
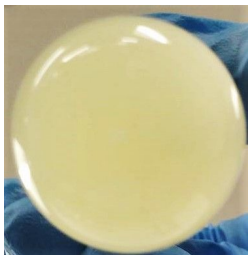
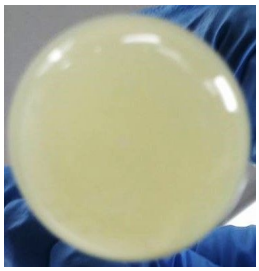
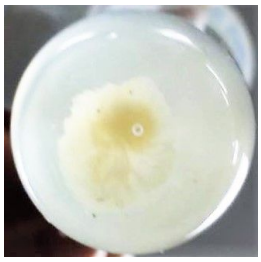
- inoculate for autoaggregation test tomorrow morning
    - *Enterobacter aerogenes*
    - DH5a w/ EC93 cosmid
    - DH5a w/ pSK33 (neg control)
    - X90 w/ EC93
    - EPI100 w/ EC869

July 13th

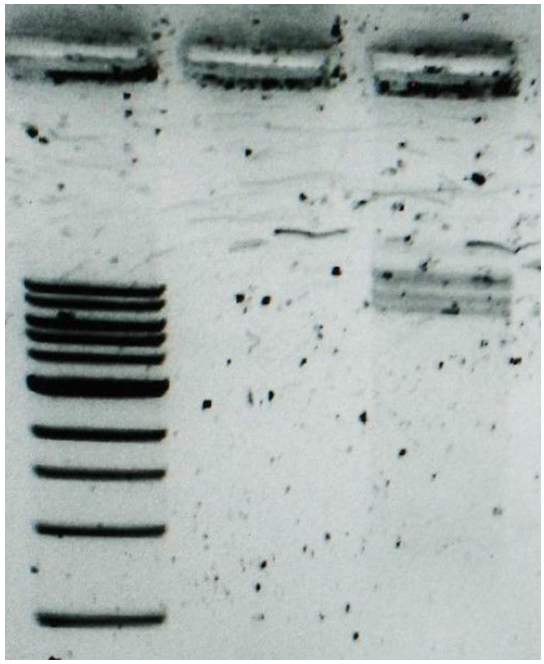
AG/OE

Aggregation test results:

Strain/Cosmid	t = 0h	t = 2h
ATCC 13048		
DH5-alpha w/ EC93		

DH5-alpha w/ pSK33		
X90 w/ EC93 **** expected aggregation****		
EPI100 w/ EC869		

- X90 w/ EC93 did not show any aggregation → mini-prepped (291.22 ng/uL) and ran test digest (1hr at 37 C, 20 min at 80C w EcoRI)



ladder test digest

- bands at 10kb and 8kb as expected (third band may be supercoiled cosmid)

<u>cPCR of DH5-alpha w/ pSK33</u>

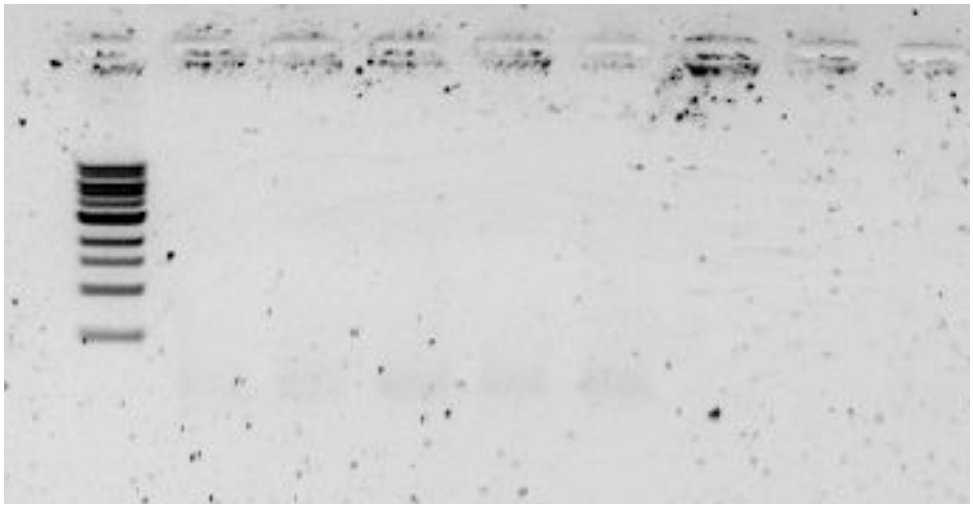
- selected 8 colonies

1uL of 10uM F primer

1uL of 10uM R primer

1 uL colony solution (pipette tip to select one colony and resuspend in 100 uL water)
25 uL 2X APEX MM
water to 50 uL

95C --- 5 min
95C --- 30s
49C --- 30s (annealing)
72C --- 10s (ext)
72C --- 3min



ladder/ colony 1/ 2/ 3/ 4/ 5/ 6/ 7/ 8

- nothing showed up on the gel
    - used incorrect CPCR primers
- reinoculated X90 w/ EC93 to redo auto-aggregation assay

OE

- Categorized CDI primers that had already been ordered into usable and unusable groups bc many had been designed improperly
- Worked on designing all new gibson primers for CDI constructs

AC

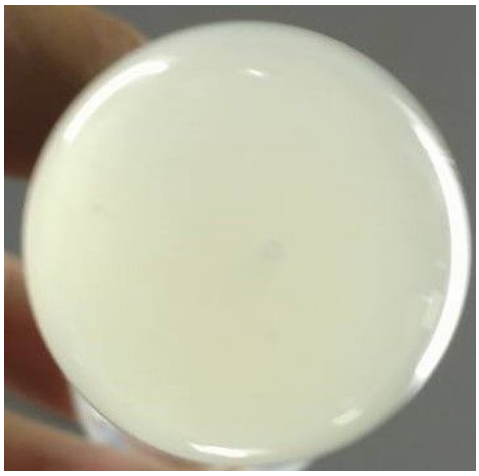
Prepared materials needed Lambda Red CdiA knockout

- Obtained pTKRed plasmid
    - Spectinomycin resistance
- Prepared spectinomycin agar plates
    - 12.5g LB
    - 7.5g Bacto-Agar
    - 500mL dH2O
    - 500uL Spectinomycin
    - Poured into miniplates
- Prepared 800mL of autoclaved Milli-Q water
    - 2 bottles of 400mL
    - Autoclaved in liquid cycle
- Prepared autoclaved 250mL flask
- Ordered primers needed to make KanR genes
    - 50bp homology + KanR + 50bp homology
    - Homology regions come at beginning of CdiA and end of CdiA
        - Doesn't include CT or Cdi-I regions
    - Ordered for EBL1 and EBL2

July 14th

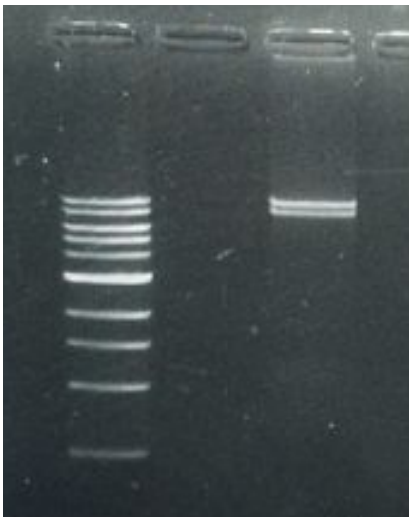
AG

- no aggregation observed in EC93 culture → prepare for competition assay to test for proper CDI complex expression



X90 w/ EC93; t = 2h

- redoing test digest → made glycerol stock (LABEL: X90 EC93 7/14/16)



- replate DH5-alpha pSK33 on new kan plate for single colonies
- plated DH5a w/ Loci 2 pSB1C3 (1:1)
- plated DH5-alpha w/ EC869 (1:1, 1:100, 1:1000)

AC

- gibson assembly of pSB1C3 loci 2
    - Fragment concentrations
        - EBL2 frag 2: 19.3ng/uL
        - EBL2 frag 3: 65.85ng.uL
            - 2 fold dilution for 32.925ng/uL
        - EBL2 frag 4 (pSB1C3 Backbone): 22.83ng.uL
        - EBL2 frag 1: 24.94 ng/uL
    - Add all fragments in equimolar amounts and fill up to 5uL of DNA mix
        - EBL2 frag 1: 0.775uL
        - EBL2 frag 2: 1.3uL
        - EBL2 frag 3 (diluted): 0.147uL
        - EBL2 frag 4: 0.282uL
        - dH2O: 2.35uL
    - Add in Mastermix and incubate at 50C for 1hr

        - 5uL DNA mix + 5uL GA HiFi 1-Step Mastermix (2x) on ice
        - Incubate at 50C for 1 hr
- PCR purification (elute into water)

OE
- transform DH5-alpha with pSB1C3 loci 2
- transform DH5-alpha with EC869 cosmid

July 15th

AG/OE
- ordered cPCR primers for pSK33 (the other primers used in the cPCR were for EC93 cosmid accidentally)
- no autoaggregation in Enterobacter observed

<u>EBL1 Toxin Switch (TS)</u>

Q5 protocol
1.25uL of 10uM F primer
1.25uL of 10uM R primer
10ng/20ng gDNA
12.5uL Q5 2X MM
water to 25uL

initial denaturation: 98 C --- 30s
30 cycles: 98 C --- 10s
Tm --- 30s
72 C --- 20-30s/kb
final ext: 72C --- 2 min
Hold: 10 C --- infinite

Fragment 1 (8kb)
- anneal at 64 C
- 72 C for 4 min

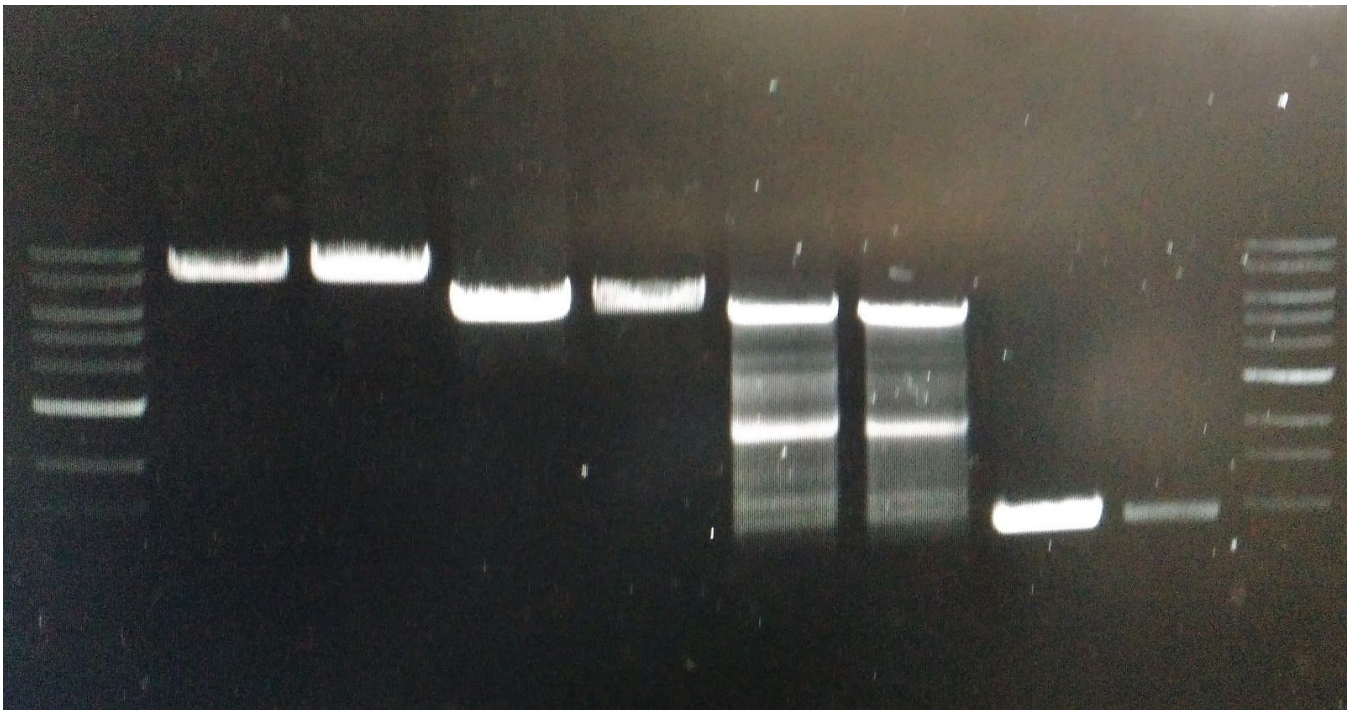
Fragment 2 (pSK33)
- digest with EcoR1 and BamH1
    - couldn't do bc not enough pSK33

Fragment 3 (5.8kb)
- anneal at 63 C
- 72 C for 2 min 54 s

Fragment 4 (5.6kb)
- anneal at 65 C
- 72 C for 2 min 48 s

Fragment 5 (900bp)
- anneal at 67 C
- 72 C for 28 s

- run all fragments on gel (except frag 2-- pSK33) and gel purify successful products



lanes: ladder/ frag 1 (10ng)/ 1 (20ng)/ 3 (10ng)/ 3 (20ng)/ 4 (10ng)/ 4 (20ng)/ 5 (1ng);/5 (5ng)

- frag 1, 3, and 4 are from Enterobacter gDNA; frag 5 from EC93 cosmid
- smearing in band 4 probably due to nonspecific primer binding to other regions of gDNA
- gel purified all fragments and combined bands of the same fragment
    - frag 1: 63.11 ng/uL
    - frag 3: 77.48 ng/uL
    - frag 4: 63.87 ng/uL
    - frag 5: 110.34 ng/uL
    - all 260:280 between 1.8 and 1.9

- incoluation for test digests, making electrocompetent cells and cloning more pSK33:
    - 5mL DH5a w/ EC869
    - 5mL pSB1C3 w/ Loci 2
    - 5mL Enterobacter
    - 10 mL DH5a w/ pSK33

AC

- Over-grew Enterobacter (went from 0.116 to 1 in 2.5h)
    - Initially added 0.5mL overnight culture to 50mL of LB in 37C shaker

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July 16th

AG

- observed growth in DH5a w/ pSB1C3 EBL2
- observed growth in DH5a w/ EC869
    - made glycerol stocks
- test digest:
  pSB1C3 EBL2 (8kb, 7.6kb, 4kb)
    - XmaI: 1uL (37 for 1h, 65 for 20min)
    - BsaI: 1uL (37 for 1h, 65 for 20min)
    - H2O to 50 uL (37.6uL)
    - 500 ng DNA = 5.44 uL sol'n (91.82ng/uL)
    - 5uL cutsmart buffer

EC869 cosmid (10kb, 8kb)

- EcoR1: 1uL
- H2O to 50 uL (42.6)
- 500 ng DNA = 1.45 uL sol'n (344 ng/uL)
- 5uL NEBuffer 2.1

- mini-prepped DH5a-pSK33 (66.45 ng/uL)
- test digest pSK33
  - 1uL EcoR1 (37 1h, 65 20min)
  - 1uL BamH1-HF (37 1h)
  - H2O to 50uL (33uL)
  - 10 uL DNA
  - 5uL NEBuffer 2.1
- PCR of flag tag fragments (4 and 5)

<u>Q5 protocol</u>
1.25uL of 10uM F primer
1.25uL of 10uM R primer
10ng gDNA or 1ng of EC93 cosmid
12.5uL Q5 2X MM
water to 25uL

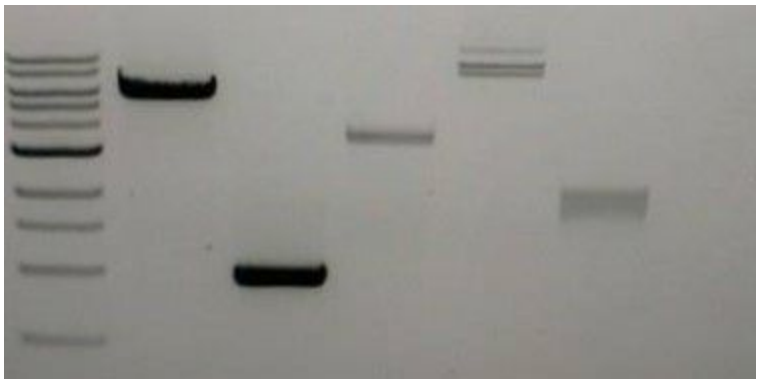
initial denaturation: 98 C --- 30s
25 cycles: 98 C --- 10s
Tm --- 30s
72 C --- 20-30s/kb
final ext: 72C --- 2 min
Hold: 10 C --- infinite

Fragment 4 (5.6kb)

- anneal at 65 C
- 72 C for 2 min 48 s

Fragment 5 (900bp)

- anneal at 67 C
- 72 C for 27 s



4FT 5FT pSK33 EC869 pSB1C3 w Loci 2

- gel purified pSK33 (17.93 ng/uL bad 260:280); frag 4 FT (58.81ng/uL); frag 5 FT(106.3 ng/uL)
  - pSB1C3 wrong size
- inoculate more Enterobacter/DH5-a with pSK33 (2x 10mL)

AC/AG

- made electrocompetent Enterobacter cells
  - Inoculate overnight culture
  - Add 0.5mL of overnight culture to 50mL of LB in a 250mL flask

    - Incubate in shaker at 37C
    - Take OD600 after 1 hour
        - Continue taking at around 15min intervals until reach an OD of 0.4-0.6
    - Remove from shaker and transfer into two 50mL plastic Falcon tubes (25mL of liquid culture in each
    - Immediately place tubes into ice-water slurry and mix tubes and ice for 4-5min
        - Move to cold room to perform other steps
        - Cells must be kept at 4C or below until transformation
    - Centrifuge Falcon tubes at 3000rpm (~3000xg) for 3min at 4C
        - Use large centrifuge next to incubator
    - In cold room, remove LB without disturbing pellet
    - Add in 50mL of autoclaved Milli-Q Water per tube
        - Repeat above step 2 **MORE TIMES**
    - Resuspend cells in 5mL of Milli-Q water
    - Aliquot into 30uL volumes for transformation
- transformed with pTKred
    - no cells (arc time 6.0 and no pellet from centrifugation)
    - most likely cells were too dilute
        - Perhaps try final resuspension in 100uL of water instead of 5mL of water

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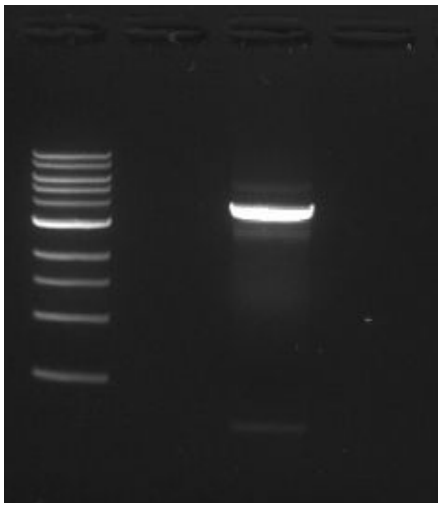
July 17th

AG

- mini-prepped two 10mL cultures of DH5a w/ pSK33
    - 80.25 ng/uL and 66.34 ng/uL
- digest of pSK33 to linearize it for gibson
    - 1uL EcoR1 (37 1h, 65 20min)
    - 1uL BamH1-HF (37 1h)
    - H2O to 50uL (28uL; 30.5uL)
    - 1.5 ug DNA
    - 5uL NEBuffer 2.1
    - 2 hr at 37 C → immediately gel purify (BamH1 cannot be heat inactivated)
        - expect band at 4kb



ladder/ pSK33

- PCR PURIFY RIGHT AWAY (to get rid of BamH1 enzyme)
    - don't nanodrop
- gel purify fragments (15uL sample, 5uL dye, 10 uL H2O)
    - pSK33 = 43.55 ng/uL
- Gibson Assembly (no FT)
    - Frag 1: 0.40 uL (25 ng)
    - Frag 2: 0.26 uL (11.21 ng)

    - Frag 3: 0.23 uL (17.8 ng)
    - Frag 4: 0.27 uL (17.2 ng)
    - Frag 5: 0.13 uL (13.8 ng)
    - H2O: 3.82 uL
- Gibson Assembly (FT)
    - Frag 1: 0.40 uL (25 ng)
    - Frag 2: 0.26 uL (11.21 ng)
    - Frag 3: 0.23 uL (17.8 ng)
    - Frag 4: 0.27 uL (17.2 ng)
    - Frag 5: 0.13 uL (13.8 ng)
    - H2O: 3.82 uL

1. thaw GA MM on ice
2. put fragments and H2O in PCR tube
3. combine 5uL fragments and 5uL MM → pipette 3 times to mix
4. incubate at 50 C for 1 hr
5. PCR purify into water (10 uL)
6. use 2uL to transform cells
    a. plated 1:1 and 1:100 of both pSK33-TSL1 with and without flag tag (on kan plates)

OE

- the psk33 w/ Aerogenes Loci 2 [EC93 Toxin] and Flag Tag Benchling files did not have the attached primers after the CDI-I region that was necessary for proper amplification. Luckily some of the old psc101 primers work.
    - 1F_EBL2-p33-TS_GP
    - 1R_EBL2-p1c3_GP
    - 2F_EBL2-p1c3_GP
    - 2R_EBL2-p33-TS_GP
    - 3F_EBL2-p33-TS_GP
    - 3R_EBL2-p101-TS_GP
    - 3R_EBL2-p33-TS_GP
    - 4F_EBL2-p101-TS_GP

1.25µL forward primer (to get 500nM)
1.25µL reverse primer
10ng of DNA insert
12.5 µL of Q5 enzyme (for 2X reaction)
H2O up to 25µL

<u>PCR fragments:</u>
Fragment 1: 1F_EBL2-p33-TS_GP/1R_EBL2-p1c3_GP
Anneal at 65°C and extend for 3 minutes and 22 seconds

Fragment 2: 2F_EBL2-p1c3_GP/2R_EBL2-p33-TS_GP
Anneal at 66°C and extend for 3 minutes

Fragment 3: 3F_EBL2-p33-TS_GP/3R_EBL2-p101-TS_GP
Anneal at 66°C and extend for 32 seconds

Fragment 4: 4F_EBL2-p101-TS_GP/3R_EBL2-p33-TS_GP
Anneal at 64°C and extend for 2 minutes and 42 seconds

initial denaturation: 98 C --- 30s
25 cycles: 98 C --- 10s
Tm --- 30s
72 C --- 20-30s/kb
final ext: 72C --- 2 min
Hold: 14 C --- infinite

July 18th

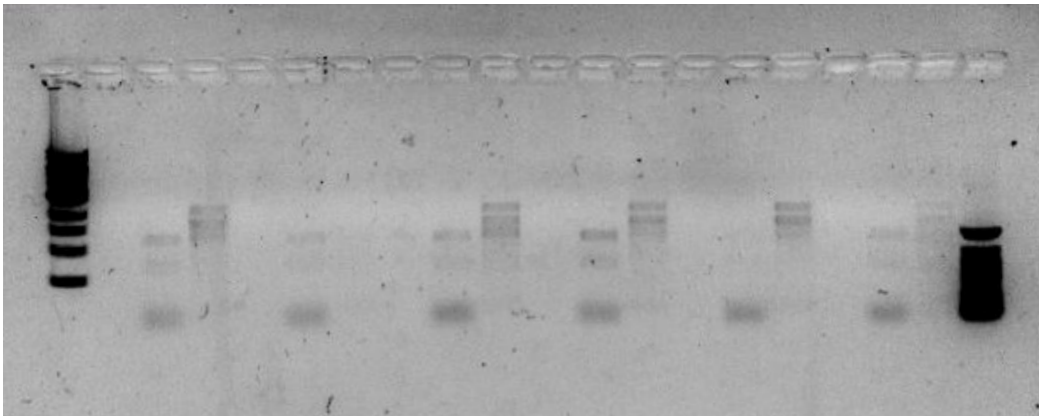
AG

- colonies grew on both 1:1 and 1:100 plates for both DH5a w/ EBL1 TS w/o flag tag and with flag tag
    - cPCR (6 colonies, 3 CPCRs each)

1uL of 10uM F primer
1uL of 10uM R primer
1 uL colony solution (pipette tip to select one colony and resuspend in 100 uL water)
25 uL 2X APEX MM
water to 50 uL (22uL)

95C 5 min
95C 30s
Tm 30s (annealing)
72C 10s (ext)
72C 3min
12C hold

- Region 1 (199bp) red
    - 51 C annealing temp
- Region 2 (193bp) blue
    - 52 C
- Region 3 (140bp) green
    - 50 C annealing temp
- CPCR failed (no bands in the right place → try test digest of 4x 10mL cultures)

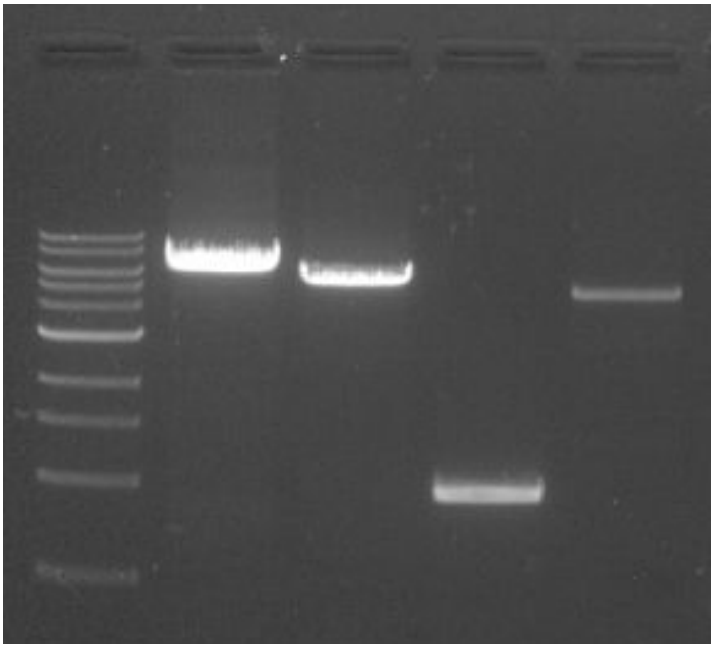


ladder (1kb)-- accident, C1R1, C1R2, C1R3, C2R1, C2R2, C2R3, C3R1, C3R2, C3R3, C4R1, C4R2, C4R3, C5R1, C5R2, C5R3, C6R1, C6R2, C6R3, ladder (50bp)

- inoculated colonies 1, 3, 4 and 5 (5 had the best cPCR result)

OE

- Performed PCR twice, both failed… Do not use bio-rad pcr tubes in ProFlex because the tube caps will come off
- Redoing PCR with original tubes



ladder/ EBL2 frag 1/ frag 2/ frag 3/ frag 4

- Gibson of EBL2
    - F1 PCR: 77.99 ng/µL 2.41 260/280
    - F2 PCR: 42.73 ng/µL 3.41 260/280
    - F3 PCR: 50.57 ng/µL 2.47 260/280
    - F4 PCR: 24.91 ng/µL 4.07 260/280
- Use 25ng for biggest frag (F1): 0.33 µL
- Use 22.2ng for 2nd largest frag (F2): 0.52 µL
- Use 20.05ng for 3rd largest frag (F4): 0.81 µL
- Use 14.9ng for 4th largest frag (pSK33): 0.35µL
- Use 18.64 ng for 5th largest frag (toxin/cdiI): 0.37µL
    - 2.62 H2O
- Run at 50°C for an hour

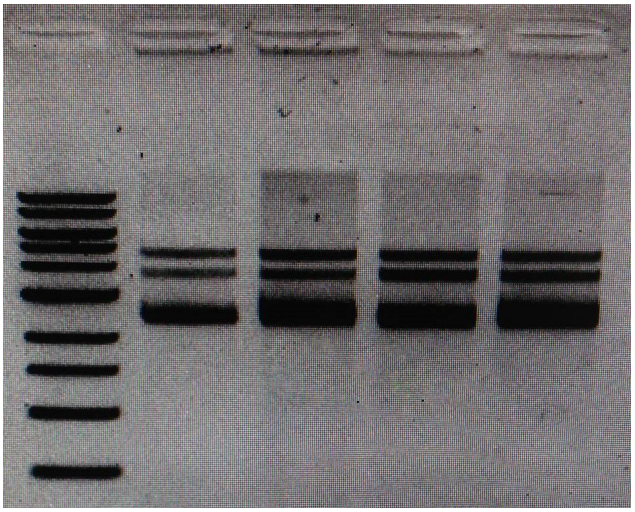
- Preparation of electrocompetent cells (E. Aero)
    - Innoculate culture
    - Add 0.5 mL of overnight culture to 50 mL of the LB in a 250 mL flask
    - Incubate @37 C in shaker
    - Take OD600 after 1 hour
        - Continue taking @ around 15 min intervals until you reach an OD of 0.4-0.6
    - Remove from shaker and transfer into two 50 mL plastic falcon tubes (25 mL of liquid culture in each)
    - Immediately place tubes into ice-water slurry and mix tubes and ice for 4-5 minutes
        - Move to cold room
        - Cells must now be kept @ 4 C or lower for transformation
    - Centrifuge falcon tubes at 3000 rpm (~3000g) for 3 minutes @ 4 C
    - In cold room, remove LB without disturbing pellet
    - Add in 50 mL of autoclaved Milli-Q water per tube
        - Repeat last two steps two more times
    - Resuspend cells in 100 µL of Milli-Q water
    - Aliquot into 30 µL volumes for transformations
- Transform lambda-red

July 19th

AG

- mini-prepped colonies 1, 3, 4, and 5

- test digest (expected frag: 11kb, 5.6kb, 4kb, 2kb, 1.5kb)
    - 1uL XmaI
    - 5uL cutsmart buffer
    - 1ng DNA
    - H2O up to 50uL
- digest for 1.5 hr and run the gel at 95V for 1hr



ladder, colony 1, 3, 4, 5

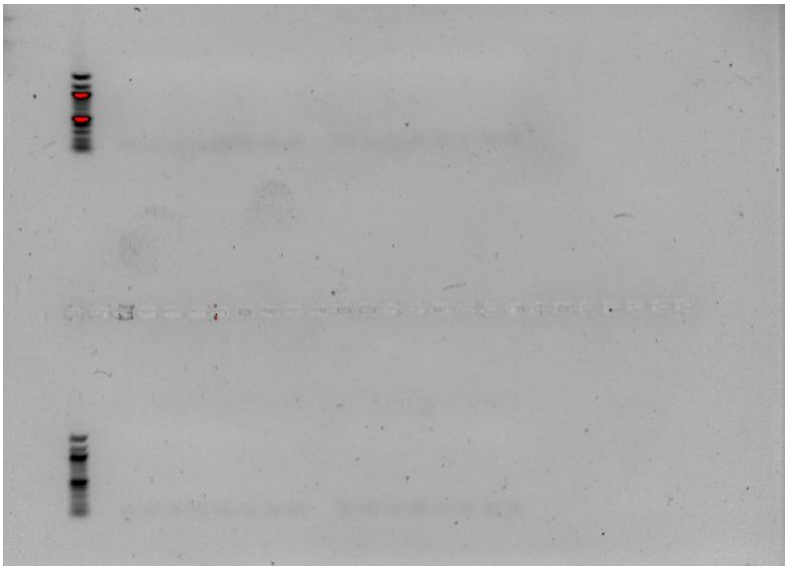
AC/OE

- Re tried making competent cells
    - Use overnight culture and take OD 600
        - Add 0.5mL culture to 50mL LB and incubate at 37C
        - 0.416 OD after 1hr and 50min
        - Transfer into 2 falcon tubes of ~25mL each
        - Swirl falcon tubes in ice-water slurry for 5min
        - Spin at 3000g for 5min (no pellet collected)
            - Spin again at 3000g for 5min (no visible pellet)
        - Removed supernatant
        - Replaced with 25mL of Milli-Q Water
        - Spin at 3000g for 5min
            - No pellet collected again so assumed failure

July 21st

AG

- cPCR region 1 on 32 colonies
    - H2O to 25 uL (11uL)
    - 0.5uL forward primer
    - 0.5uL reverse primer
    - 1uL colony suspension
    - 12.5uL APEX master mix
- master mix cPCR:
    - 460uL H2O
    - 20uL forward primer
    - 20uL reverse primer
    - 500uL APEX
    - put 24 uL of the MM into each PCR tube and 1 uL of the colony suspension



ladder/ colony 1/ 2/ 3/ 4/ 5/ 6/ 7/ 8/ 9/ 10/11/ 12/ 13/ 14/ 15/ 16
ladder/ colony 17/ 18/ 19/ 20/ 21/ 22/ 23/ 24/ 25/ 26/ 27/ 28/ 29/ 30/ 31/ 32

OE/ AC

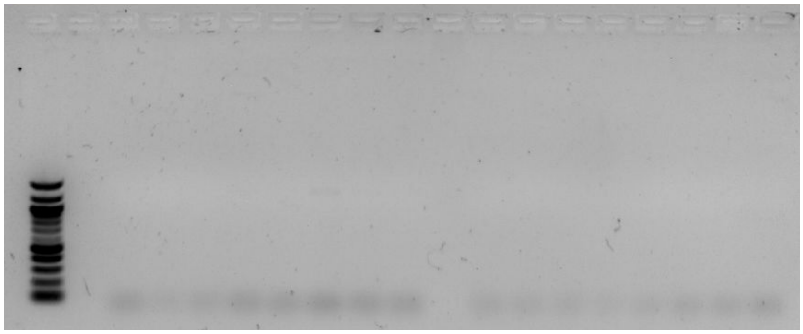
- Remade enterobacter aerogenes
- Changes:
    - Spun @ 8000 rpm for 10 minutes
    - Last pellet was very soft and first two were very hard
    - Resuspending in 100µL was difficult because pellet was disturbed too easily
    - When transforming, the cells looked like they weren't very concentrated
    - Arc time was 5.9 and we accidentally put the heat sensitive lambda red transformations in the shaker @ 37 C for 10 minutes as opposed to the 30 C
    - We also ran out of normal sized petri dishes so we had plated onto tiny Kan-R plates with ¼ the surface area of a normal petri dish

---

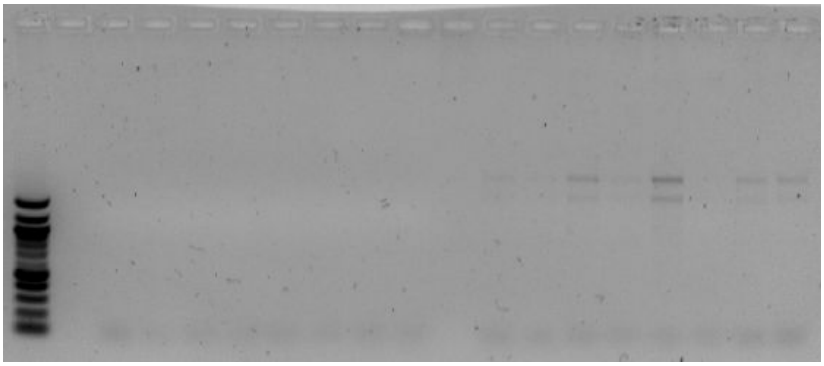
July 22nd

AG

- CPCR of regions 2 and 3 with colonies 1-16
    - Region 2 (193bp) blue
        - 52 C
    - Region 3 (140bp) green
        - 50 C annealing temp
    - ladder (100bp)
        - 14uL water
        - 1uL ladder
        - 3uL dye
    - ran at 100V for 30 min (last time had poor resolution)
        - no positive results (probably primer dimers)



ladder/ cpcr region 2 colony 1/ 2/ 3/ 4/ 5/ 6/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 15/ 16



ladder/ cpcr region 3 colony 1/ 2/ 3/ 4/ 5/ 6/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 15/ 16

- Gibson re-do of EBL1 TS pSK33
    - .004794 pmol
    - frag 1: 25 ng = 0.396 uL x
    - frag 2: 11.199 ng = 0.257 uL x
    - frag 3: 17.827 ng = 0.230 uL x
    - frag 4: 17.175 ng = 0.269 uL
    - frag 5: 13.825 ng = 0.125 uL
    - H2O = 3.723 uL x

OE

- Nothing grew on the transformed lambda-red plates
    - Potential problems:
        - Arc time
        - Cells were too dilute
        - Washed away too many cells
        - Bad incubation
- Going to redo the whole process but after second spin of milli-q, put the solution in 1.5 mL tubes and spin @ max speed
- CPCR Protocol:
    - Take colony and resuspend in 100µL of dH2O
        - Prepare 16 colonies
    - Add
        - H2O to 25 µL
        - 0.5 µL forward primer
        - 0.5 µL reverse primer
        - 1 µL colony suspension
        - 12.5 µL APEX master mix
    - Cycle
        - 95 C for 5 minutes
        - 25 x
            - 95 C for 30 seconds
            - Annealing Temp for 30 seconds
            - 72 C for extention time
        - 72 C for 1 minute
        - 12 C infinite hold
- F1: length 360 bp anneal @ 50 C extention time 14.4 seconds
- F2: length 297 bp anneal @ 49 C extention time 11.88 seconds
- F3: length 332 bp anneal @ 50 C extention time 13.28 seconds

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July 23rd

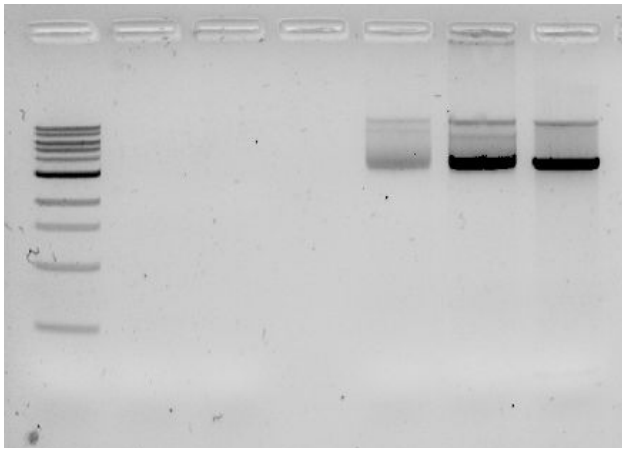
OE

- Enterobacter still has not grown
- We believe that the issue with the digestions performed on our backbone vectors were done incorrectly because we had used both high fidelity enzyme (BamI) and a non high fidelity enzyme (EcoRI). But these require different buffers to operate whereas we had used both enzymes in the buffer that is suitable only for HiFI enzymes.
- Performed digestions over again

July 24th

AG

- inoculated 4x 10mL cultures of DH5a w/ pSK33 (7/23/16)
    - miniprepped all of them
- digest of pSK33 probably didn't work (used BamHI-HF instead of BamHI which has different buffer conditions → needed cutsmart but used NEBuffer 2.1 so BamHI-HF only had 50% activity)
- double digest
    - 5uL of Cutsmart
    - 1uL EcoRI-HF
    - 1uL BamHI-HF
    - 2ug DNA (30uL of pSK33 conc 67.60 ng/uL)
    - H2O up to 50uL (13uL)
    - digest for 2 hours and PCR purify after
- controls:
    - EcoRI single cutter
        - 5uL NEBuffer 2.1
        - 1uL EcoRI
        - 1ug DNA (18uL of pSK33 conc 56 ng/uL)
        - H2O up to 50uL (26uL)
        - digest 2hr and heat inactivate at 65 for 20 min
    - uncut plasmid



ladder/ uncut/ single cut/ double cut

- Gibson Assembly (no FT)
    - .004794 pmol
    - frag 1: 25 ng = 0.396 uL
    - frag 2: 11.199 ng = 0.195 uL
    - frag 3: 17.827 ng = 0.230 uL
    - frag 4: 17.175 ng = 0.269 uL

    - frag 5: 13.825 ng = 0.125 uL
    - 3.785uL H2O
    - 5uL Gibson MM
    - 50 C for 1 hr
- inoculate: DH5a w/ ampR; EC93 in EPI100; EC869 in X90; DH5a w/ pSK33; Enterobacter

OE

- PCR of Kan marker for transposon insertion doesn't seem to be working, Jared had asked me to try it on my own
    - Tried the original annealing temperature of 65 C as well as +- 3C
    - Used 23.88 seconds as the extension time
        - 1.25 µL forward primer
        - 1.25 µL reverse primer
        - 1 µL of DNA insert
        - 12,5 µL Q5 2X Master Mix
        - H2O up to 25 µL
- PCR failed again, checked primer design and noticed a presence of both secondary structures and multiple annealing regions
- Redoing Gibson Assembly with the properly digested fragments
    - Used 25ng for the largest fragment (F1)
    - 22.2 ng for F2
    - 20.05 ng for F4
    - 14.9 ng for pSK33
    - 18.64 ng for the toxin fragment

July 25th

AP

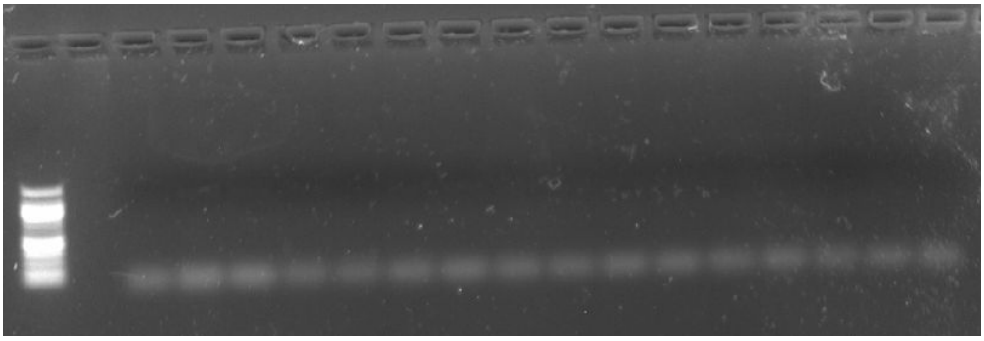
- cpcr of omar's EBL2 w/ TS no FT colonies (regions 1, 2 and 3) - 16 colonies each

AC

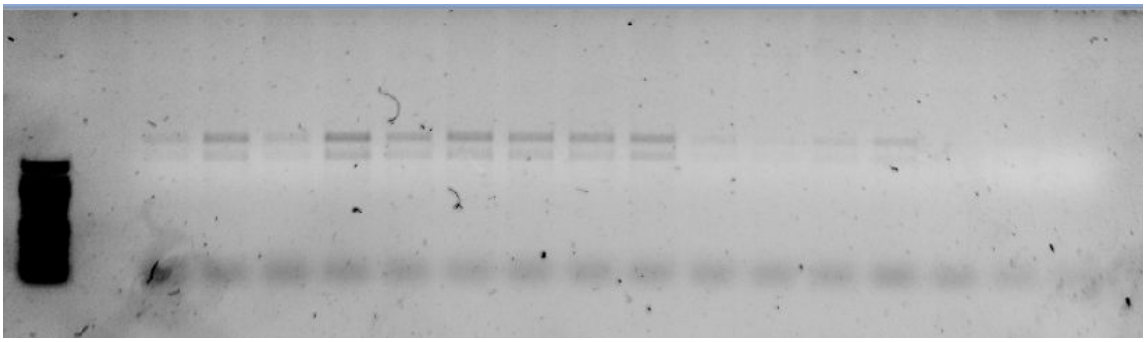
- redoing competent cells
    - used DH5-alpha control

AG

- cpcr of EBL1 w/ TS no FT (regions 2 and 3) - 16 colonies each
    - same results as before the fixed digestion
- cpcr of kan marker control
    - MM: 105 uL H2O, 5uL F primer, 5uL R primer, 125 uL APEX
    - colonies 1-8



ladder/ cpcr region 2 colony 1/ 2/ 3/ 4/ 5/ 6/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 15/ 16



ladder/ cpcr region 3 colony 1/ 2/ 3/ 4/ 5/ 6/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 15/ 16

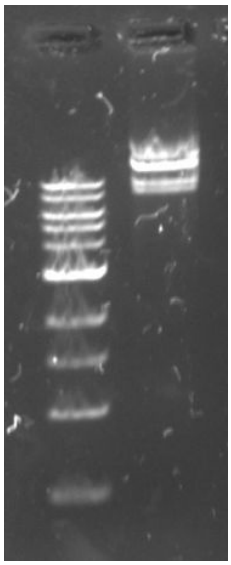
JL

- annealing temp gradient for transposon insert

July 26th

AC

- make a stock of the EPI100 w/ EC869 (500uL stock and 500uL glycerol)
- miniprep the rest of the overnight culture (4.5mL) and do a test digest:
  EC869 cosmid (expected bands:10kb, 8kb)
    - EcoR1: 1uL
    - H2O to 50 uL (42.3uL)
    - 500 ng DNA (1.7uL of a 300ng/uL stock)
    - 5uL NEBuffer 2.1
    - digest for 1hr at 37C and 65C for 20 min



ladder/ test digest

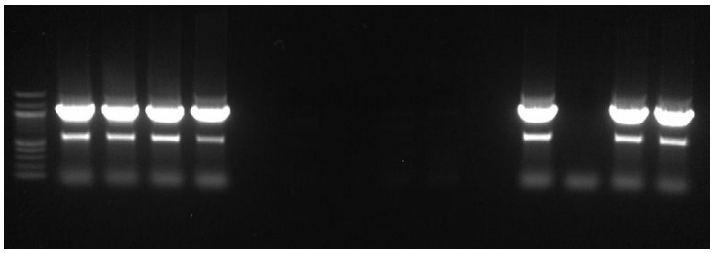
OE

- transform DH5-alpha with linearized pSK33 to check for background
    - dilute to 1ng/uL (final volume 25uL)
- transform DH5-alpha with puc19
- inoculate EPI100 w/ EC93 (also streak new plate from the stock in the -80C with a V on it for verified)
- inoculate X90 w/ EC869 (use rainin tip)
    - streak new plate if alan's test digest works
- **make stock of DH5-alpha ampR (negative control)**
- inoculate DH5-alpha w/ pSK33
- inoculate DH5-alpha w/ EBL1 TS and EBL2 TS
    - for sequencing

July 27th

AG/OE

- ran cPCR controls
    - used Danny's cpcr as a positive control to make sure our technique was ok (came back positive)
    - miniprepped transformed DH5a w/ EBL1 and EBL2 colonies and used CPCR region 1 primers → no results



ladder/ positive control colony 1/ 2/ 3/ 4/ miniprep colony 1/ 2/ 3/ 4/ 5/ 6/ 7/ 8/ positive control colony 5/ 6/ 7/ 8

July 28th

AC

- PCR Kan R for transposon insert with Jared's primers

AG/OE

- made more kan plates
- made sequencing primers for pSK33 variable region with cut sites

<u>Competition assay</u>

Purpose: determine whether the EC93 cosmid is expressed in EPI100 since it failed the autoaggregation test

Test inhibitor: EPI100 w/ CDI EC93 ampR

Positive control: Hayes strain (X90 w/ CDI EC869) ampR

Negative control: DH5-alpha ampR

Target (CDI-): DH5-alpha with empty pSK33 plasmid kanR

- all cells are in the shaking incubator in culture tubes with labels

1. take 0.5mL of the overnight culture (using p1000) and resuspend in 50 mL fresh LB w/ correct antibiotic inside autoclaved 250mL flasks already labeled with tape
2. place back in 37 C shaking incubator w/ tinfoil on
    a. **prepare the target strain 20 minutes before the controls/inhibitor**
3. **after 1 hour, take OD600 of all cells**
    a. should be all around 0.1-0.2
4. **after 10 min take another OD600 (target OD600 = 0.35)**
    a. if cells are growing at very different rates, back dilute faster growing culture to get it down to a lower OD
        i. resuspend appropriate vol of cells in 50 mL LB w/ appropriate antibiotic
5. mix inhibitor (or control) and target cell at a ratio of 10:1 (determine using growth phase/conc relation) in LB (w/o antibiotic)
    a. add target cell culture to the inhibitor culture

$$vol\ in\ inhibitor\ flask * (x\ cells/mL) = number\ of\ inhibitor\ cells\ in\ the\ culture = n$$

$$n / 10 = number\ of\ target\ cells\ needed = m$$

$$m * (1mL/x\ cells) = vol\ of\ target\ needed\ to\ be\ added$$

**the cells per mL will be different in each equation; depends on the specific OD of the culture

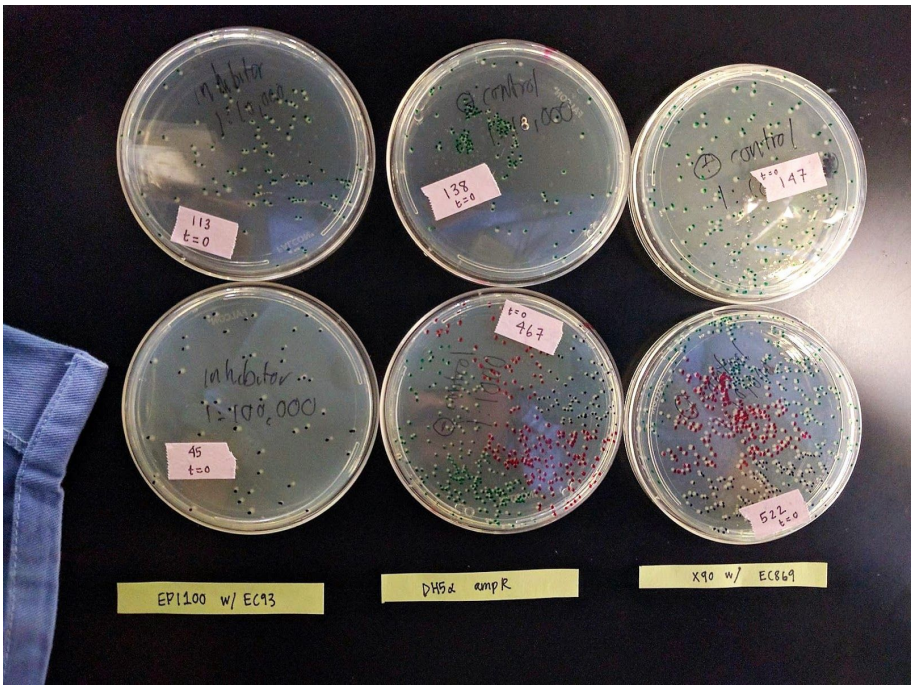
6. take a before sample (10-fold serial dilutions from 1:1 to 1:1000000)
7. incubate at 37 C shaking at 225 rpm
8. before and after sample ten-fold serially diluted in SOC (before competition assay, every hour until 4th hour, after 20 hours)
    a. plate 100uL of samples onto selective agar
    b. count CFUs per mL

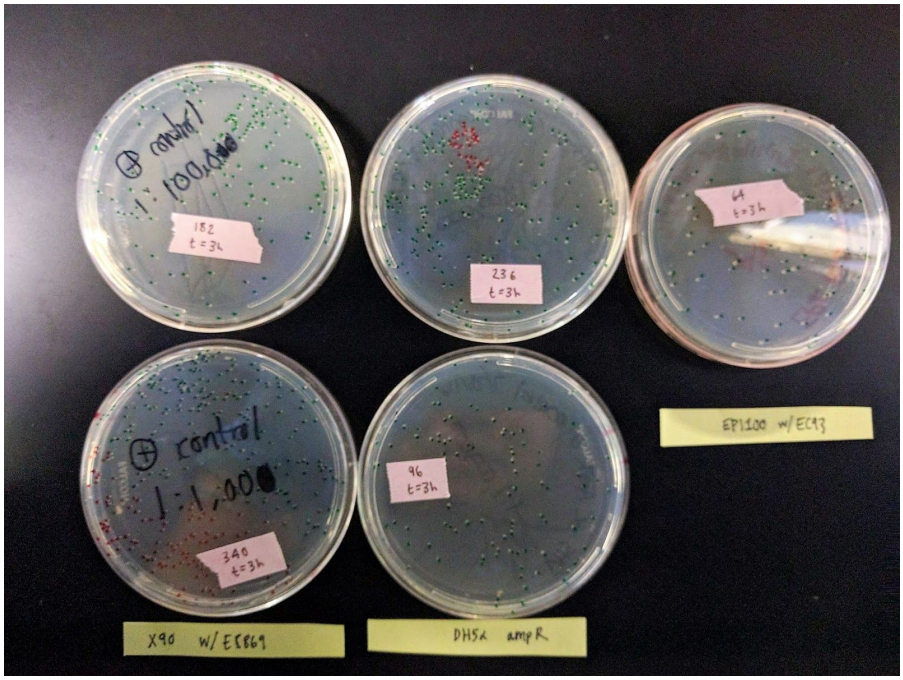
July 29th

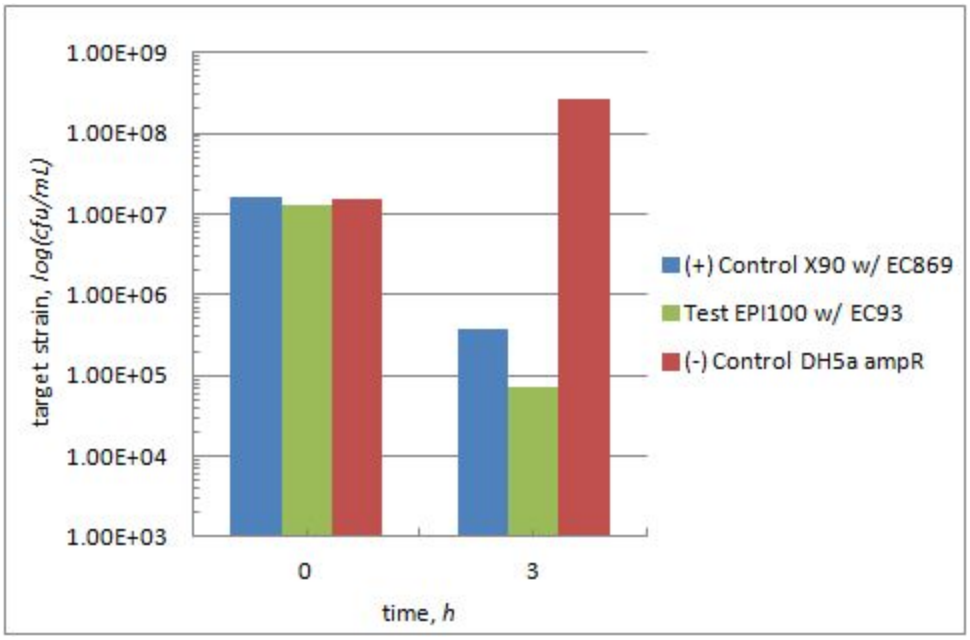
AG/OE

Results of competition assay

	CFUs/mL	
Strain	**t=0h**	**t=3h**
(+) control X90 w/ EC869	1.6 E 6	3.78 E 4
(-) control DH5a ampR	1.53 E 6	1.07 E 8
test EPI100 w/ EC93	1.26 E 6	7.11 E 3







- inoculate for new competition assay
    - DH5-a w/ EC869
    - DH5-a w/ EC93
    - DH5-a w/ ampR
    -

Notes:

- for t=0h, use 1:1000 and 1:10000 and 1:100000
- for t=0h, use 1:100, 1:1000 for inhibitors and 1:100000 and 1:1000000 for neg control

- pSK33 map was incorrect due to a 4 base pair frameshift

- order new primers for EBL1 construct w/ updated pSK33 file
- sent in pSK33 for sequencing
    - 2.5 uL of primer, 8.16uL miniprepped plasmid, 4.34uL water
    - do each primer separately
- redo kan marker cpcr on higher copy plasmid that has kan
    - found out kan marker primers oriented outward and can't be used together

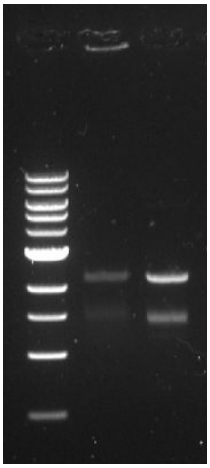
---

August 1st

AP/JL

- Transposase Activity Assay
    - 1.35 µl pSB1C3 (148 ng/µl)
    - Transposon at 92.45 ng/µl

- Gel electrophoresis
    - Control: unreacted pSB1C3

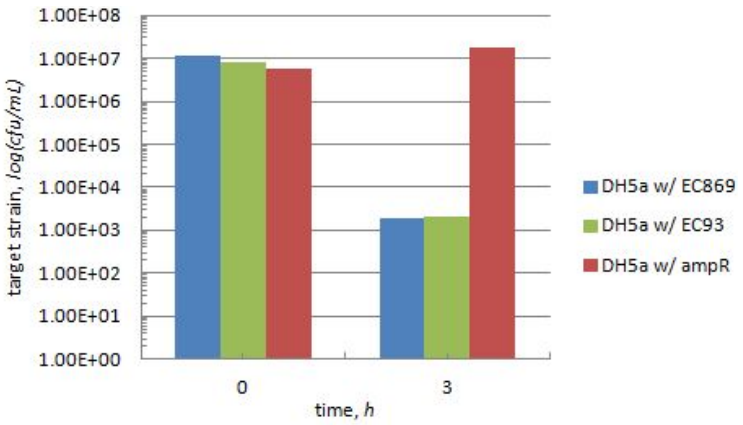


ladder/ reacted/ unreacted

AE/OE

Competition assay results

	CFUs/mL	
	t=0h	t=3h
DH5a w/ EC869	7.33 E 6	1.29 E 4
DH5a w/ EC93	4.44 E 6	2.55 E 2
DH5a w/ ampR	3.28 E 6	1.3 E 8



- inoculate same strains for another trial

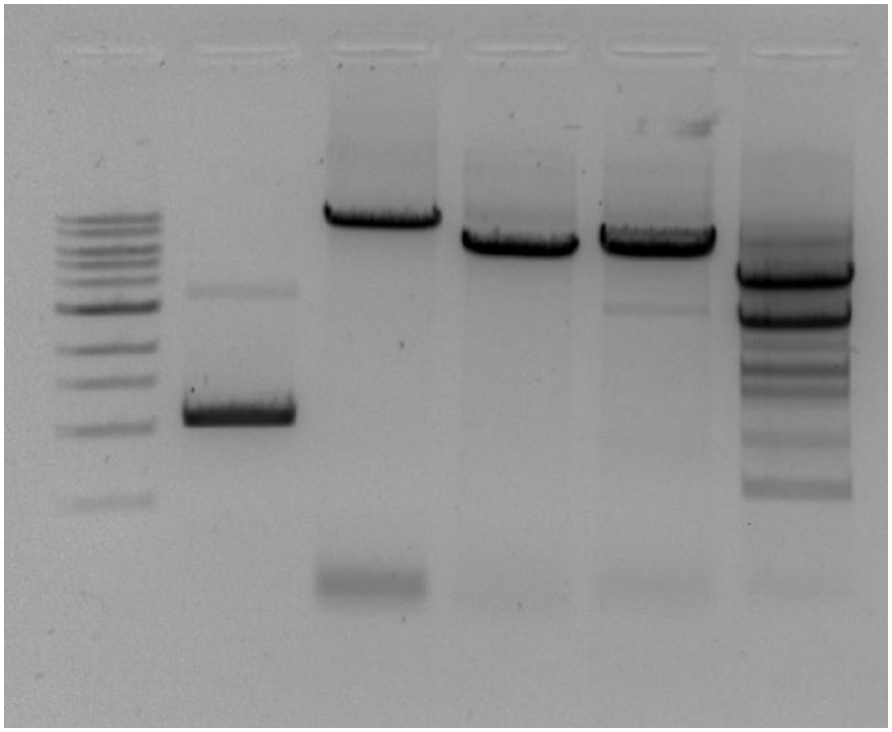
August 2nd

OE/AG

Competition Assay results

	CFUs/mL	
Strain	**t=0h**	**t=3h**
DH5a w/ EC869	1.6 E 6	3.78 E 4
DH5a w/ EC93	1.53 E 6	1.07 E 8
DH5a w/ ampR	1.26 E 6	7.11 E 3

- PCR of pSK33 backbone and frag 1 and 3 of EBL1-TS and frag 1 and 4 of EBL2-TS
    - KAPA Hifi
        - 25 uL KAPA Hifi MM
        - 1.5 uL F primer
        - 1.5 uL R primer
        - 1 ng template DNA
        - 21.0 uL H2O
        - 95 C 3 min
            - 98 C 30s
            - Tm 15s
            - 72 C 40s/kb
        - 72 C 1min/kb
        - 12 C 1hr
    - AE frag 1: 63 C, 8kb → 5 min 20s ext time
    - AE frag 3: 62 C, 5.8 kb → 3 min 52s ext time
    - pSK33: 66C, 3.3 kb → 2 min 13s ext time



Ladder pSK33 EBL1 1 EBL1 3 EBL2 1 EBL2 4

- Secondary more preferential product being formed in the PCR of pSK33 → one primer was very nonspecific (15bp homology downstream)
- EBL2 frag 4 appeared very strange -- all other bands the correct size

---

August 3rd

AP/ JL

Transposase Activity Assay 2

1. Prepare the transposon insertion reaction mixture by adding the following, in order:

1 µl EZ-Tn5 10X Reaction Buffer
0.22 µl Target plasmid (900 ng/µl)
0.67 µl Transposon (Kan R at 92.45 ng/µl)
7.11 µl sterile water (to 9 µl volume)

---

1 µl Tn5 Transposase
10 µl Total reaction volume

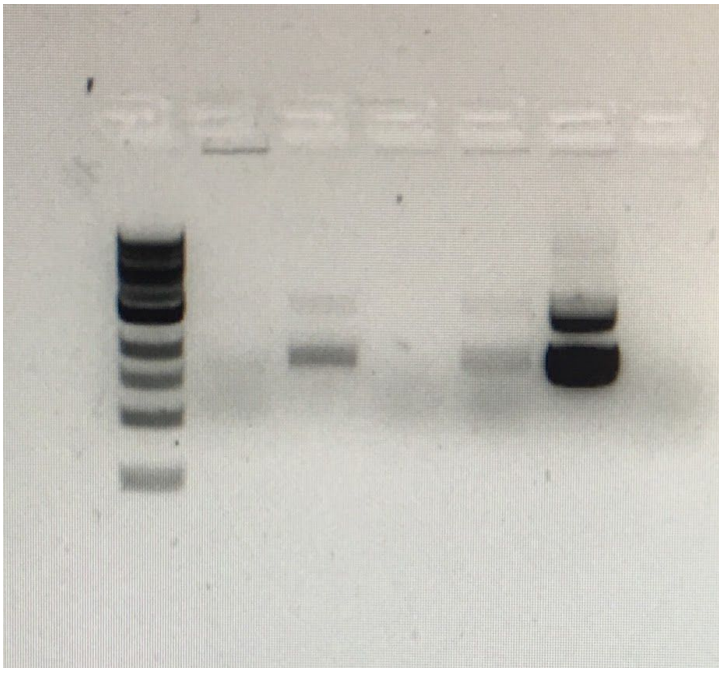
2. Incubate the reaction mixture for 2 hours at 37˚C.
3. Stop the reaction by adding 1 µl of EZ-Tn5 10X Stop Solution. Mix and heat for 10 minutes at 70°C.
   Kit: http://www.epibio.com/docs/default-source/protocols/ez-tn5-custom-transposome-construction-kits.pdf?sfvrsn=8
4. Gel electrophoresis--------> Check for transposase activity (See if transposon got inserted in plasmid)

Controls:

- Reaction without transposon
- Target reacted without transposase
- Reaction without target
- Transposon only
- Unreacted target

Lanes:

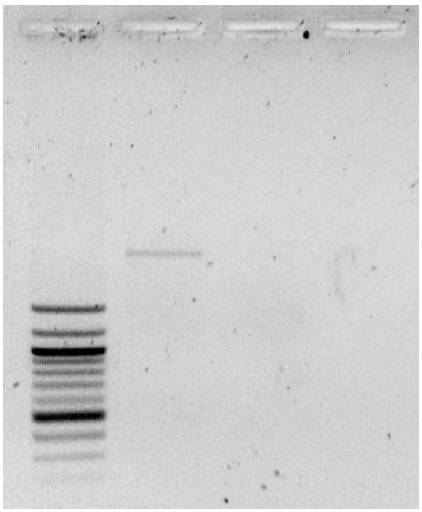
Ladder/ Reaction/ Reaction without transposase/ Reaction without transposon/ Reaction without target/ pUC19/ Transposon only



*Need to run for better resolution

AC

- PCR with positive controls
    - Tested Miniprep 1, 2 and used stock pTKRED as a positive control



ladder/ control/ miniprep 1/ 2

- Using self-designed primers
    - 1F_LBR + 1R_LBR (Random sequence on pTKRED)
    - 2F_LBR-pTKRED_cPCR + 2R_LBR-pTKRED_cPCR (On SpectR gene)
    - Mix contidions
        - 0.5uL Fwd 10nM Primer
        - 0.5uL Rev 10nM Primer
        - 1.5uL Template (133ng/ul Stock; 2ng/uL Miniprep 1; 3ng/uL Miniprep #2)
        - 12.5 OneTaq 2x Mastermix
        - 10uL Water
    - Thermocycling conditions
        - 94C--->30s
        - 28 cycles of
            - 94C--->30s
            - 52C--->30s (52C for 1F/1R; 54C for 2F/2R)
            - 68C--->30s (1min/kb)
        - 68C--->15min
        - 14C--->Infinite Hold
- Results:
    - Nothing showed up including the positive control
    - Most likely primer design problems

August 4th

JL

- PCR more transposon
- Transposase activity assay Diagnostics

AP

- Formed transposomes (using 117.57 µg/ml transposon)

OE/AG

- pSK33 and frag 4 from EBL2 gel purified (accidentally didn't add ethanol to wash buffer)
    - image saved on computer
- Re-gibsoned the constructs and transformed into DH5a

AC

- Minipreped from overnight inoculation
    - From 2 different colonies
- Ran PCR on minipreps next to stock control
    - 25uL Apex
    - 1uL Forward Primer (Calin's pTKRED Ori primers)
    - 1uL Reverse Primer (Calin's pTKRED Ori primers)
    - 23uL dH2O
    - 1uL of diluted miniprep
        - ~6ng/uL
    - Looking for a band at ~1.2kb

Results: Stock was in the correct location, minipreps did not show up

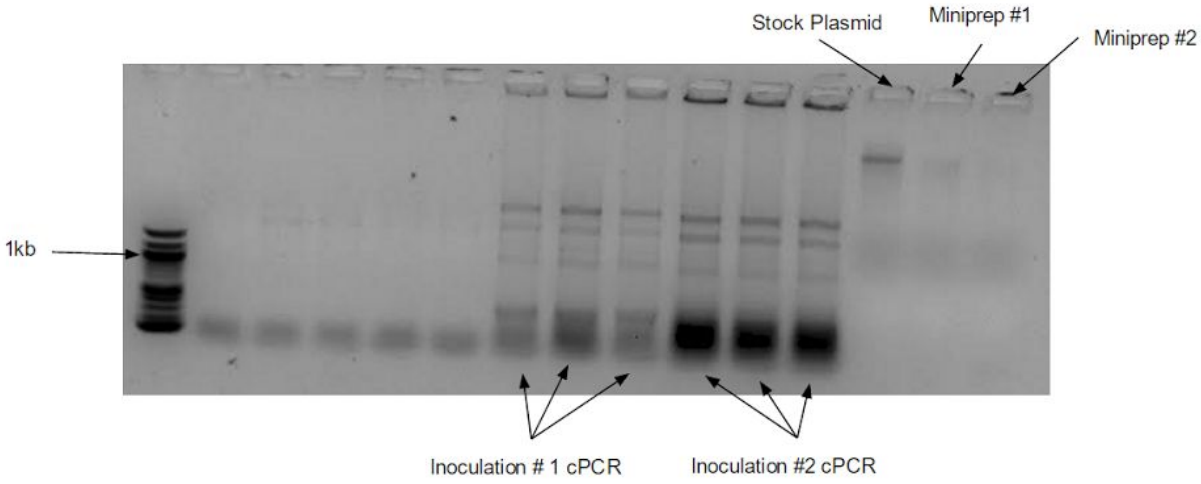
August 5th

AC

- Prepared cPCR with grown colonies (6 colonies)
    - Created mastermix with primers and Apex

- For 1 colony
  - 0.5uL of 10nM Fwd primer (Calin's Ori primer)
  - 0.5uL of 10nM Rev primer (Calin's Ori primer)
  - 12.5uL of Apex
  - 10.5uL dH2O
- Cycling Conditions
  - Initial denaturation: 3min at 94C
  - 30x of the following
    - 30s----->94C
    - 30s----->49C
    - 1:10----->72C
  - Final extension time 5min at 72C
  - Infinite hold at 14C

- Also prepared to run circular plasmid
  - Use 200ng of stock and minipreps
  - cPCR failed
    - Most likely amplified segments of gDNA
    - Very faint bands show up next to the minipreps, not entirely sure why/ what it is



OE

- Performed a CPCR of colonies grown overnight
  - Tested for the presence of at least 3 fused fragments
    - H2O up to 25 µL
    - 0.5 µL forward primer
    - 0.5 µL reverse primer
    - 1 µL colony suspension (suspended in 100 µL dH2O
    - 12.5 µL APEX
  - Cycle:
    - 95 C for 5 minutes
    - 27 X
      - 95 C for 30 seconds
      - Annealing Temp for 30 seconds
      - 72 C for extension time
    - 72 C for 5 minutes
    - 12 C hold for 1 hour
- Ran a gel with no hits....

August 7th

OE

- Theorized that the gibson had not been with enough attention to detail and stringent practice
- Redid Gibson with the same parameters but more care and attention

August 8th

AC

- Inoculated Enterobacter Aerogenes colony for use in making electro-competent cells tomorrow

AP

- Formed transposomes (stored at -20 C)
    - 2 µl Transposon DNA (117.57 µg/ml in TE Buffer)
    - 4 µl Transposase
    - 2 µl 100% glycerol
    - 8 µl Total reaction volume

OE

- Performed CPCR testing for the presence of four homology regions across 32 colonies...
    - All but two came back negative and even the two positives were not from the same colony
        - We theorized that perhaps uncut plasmid may be contaminating our samples and that may be why we are getting growth on our plates
            - Solution: DPN1 treat the backbone fragments to remove methylated plasmid (uncut plasmid):
                - Add 10 units of restriction enzyme per 1 µG of DNA insert (20 units for gDNA)
                - 1X CutSmartBuffer
                - Total reaction volume: 50 µL
                - 1hr incubation @ 37 C
                - Heat inactivate @ 80 C for 20 minutes

August 9th

AP

- Electroporated transposomes into Enterobacter
    - Added 1uL of transposomes to 50uL of homemade electro-competent Aerogenes (in 1.5 uL microcentrifuge tube)
    - Gently flicked tube to mix
    - Pipetted mixture into a chilled 10mm gap electroporation cuvette
    - Electroporated at 1.8kV
        - Arc time 3.8
    - Immediately added 950uL of SOC to cuvette
        - Pipette down, up, down, up
    - Transferred to microcentrifuge tube and shook at 800rpm at 30C for 30 min, then transferred to lightly capped culture tube at 200 rpm 37 C for 30 min
    - Centrifuged microcentrifuge tubes at 6000rpm for 4min on tabletop centrifuge
    - Discard supernatant and resuspend pellet in 50uL SOC
    - Plate 50uL of resuspended cells onto warm kan 25 plate
- Incubate overnight at 37C for 16h
- ***Need to:
    - Transform 1 µL transposomes into Dh5 alpha
    - Transform 1 µL pUC19 into Dh5 alpha (negative control)
    - Transform 1 µL pUC19 into Enterobacter (calculate transformation efficiency)

AC

- Made competent Enterobacter aerogenes
    - Take overnight culture and add 0.5mL of culture into 50mL of LB in a 250mL flask
        - Did twice, so have 2x 50mL of LB shaking in 37C
    - Let cells grow until reached an OD of 0.497
    - Immediately place flasks into ice-water slurry and swirl gently for ~5min
    - Moved everything to the cold room at 4C
        - All following steps were performed at 4C or lower
    - Aliquoted culture into 4x 50mL Falcon tubes
    - Spun tubes at 5000rpm on floortop centrifuge
    - Removed LB supernatant
    - Resuspended all pellets in 12.5mL Milli-Q Water

    - Combined suspensions into 2 Falcon tubes (final volume in each was 25mL)
    - Spin tubes at 5000rpm on floortop again
    - Discard supernatant and resuspend in 25mL Milli-Q Water
    - Repeat spin cycle and discard supernatant, but resuspend in 1mL of Milli-Q Water
    - Transfer to microcentrifuge tubes
    - Spin microcentrifuge tubes at 5000rcf on tabletop centrifuge
    - Discarded supernatant and resuspended in 30uL of Milli-Q water
- Transformation
    - Added 2uL of 1.33ng/uL pTKRED solution to 30uL of homemade electro-competent Aerogenes (still in microcentrifuge tube)
    - Lightly mixed by flicking bottom of tube
    - Pipet mixture into a chilled electroporation cuvette (between the 2 metal plates)
    - Electroporate at 1.8kV
        - Arc time was 5.5
    - Immediately add 970uL of SOC to cuvette
        - Pipette down-up twice
    - Transfer to microcentrifuge tube and shake at 800rpm at 30C for 1hr
    - After 1hr, spin microcentrifuge tubes at 6000rpm for 4min on tabletop centrifuge
    - Discard supernatant and resuspend pellet in 50uL SOC
    - Plate 50uL of resuspension onto warmed up SpecT 25 plate
    - Incubate overnight at 30C

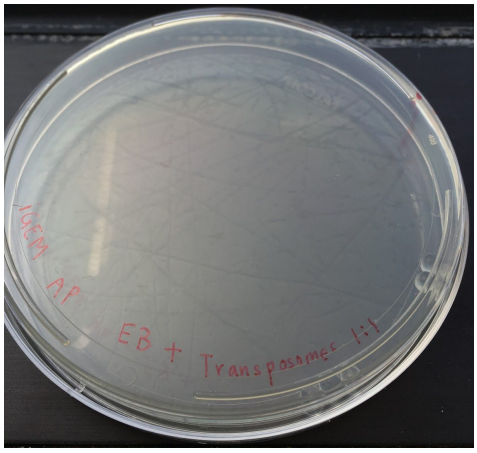
AG/OE:

- CPCR of region 1, 2, 3 and kan marker control of colonies (DH5a transformed with EBL1 TS and EBL2 andTS)
- Only one positive result for region 1 DH5a w/ EBL1 TS (neg for other two regions)
    - Inoculated for sequencing the next day

August 10th

AP

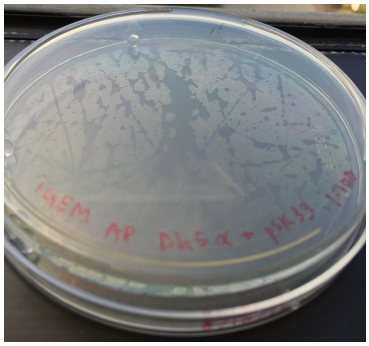
- No colonies after 16 h

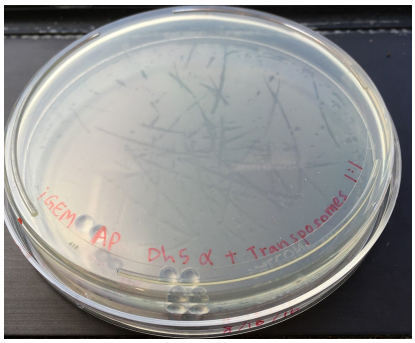


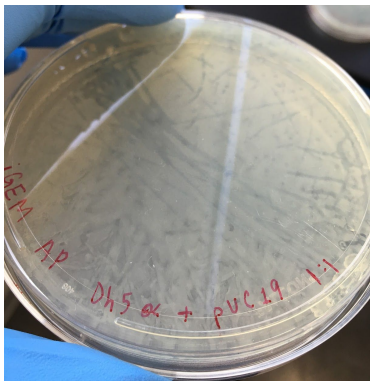
18 h

- Transformed:
    - Dh5 alpha w/ transposomes
    - Dh5 alpha w/ pUC19 (negative control)
    - Dh5 alpha w/ pSK33 (positive control) (arctime 5.3)
        - 1.0-mm gap cuvette, 1.8 kV

Results from transformation w/ Dh5 alpha:16 h







No growth on all except Negative control was a lawn because flipped positive and negative control labels. Positive was actually w/ pSK33.

AG/OE

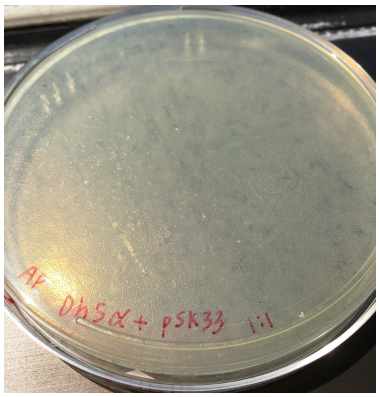
- Sequenced pSK33 w/ frag 1 attached
- Re-PCR'd pSK33, EBL1 frag4 and EBL1 frag 5 (Omar re-PCR'd all the EBL2 fragments)
    - dpnI treat the pSK33 after gel purification to cleave any leftover methylated template DNA

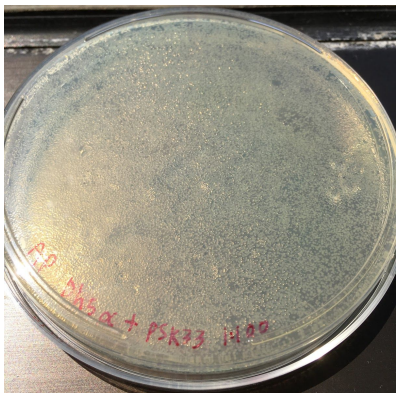
August 11th

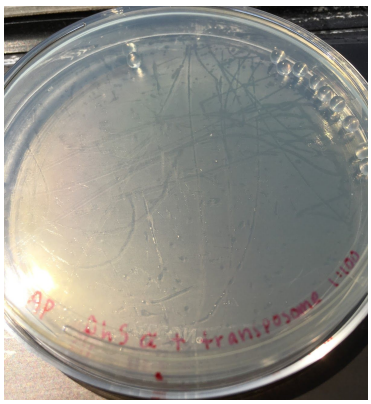
AP

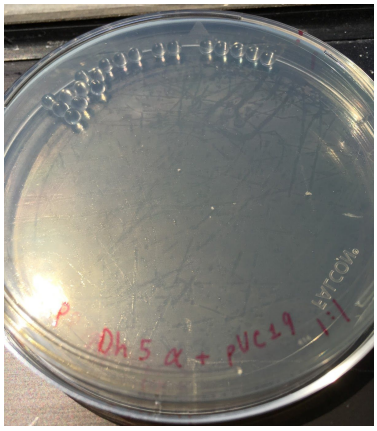
- Drop dialysis of transposase
- Formed transposomes with desalted transposases
- re-Transformed:
    - Dh5 alpha w/ transposomes (arctime 5.1)
    - Dh5 alpha w/ pUC19 (negative control) (arctime 5.00)
    - Dh5 alpha w/ pSK33 (positive control) (arctime 5.10)
        - 2.0-mm cuvette, 2.5 kV

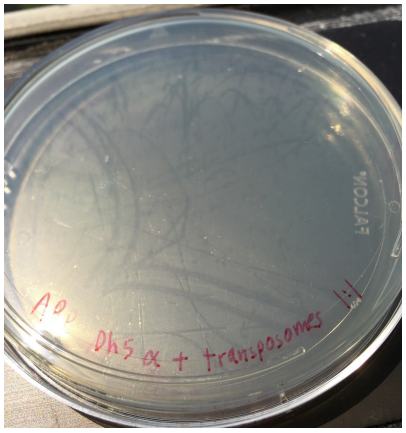
No growth on transposome plates. Positive control fine.











AG

- Sent in (+) CPCR region 1 colony for sequencing using 12F_EBL1-p33-TS_SQ and 21F_EBL1-p33-TS_SQ → no priming
- Gel purified fragment 4 for EBL1
- DpnI treated pSK33 gel purified product and PCR purified
    a. 9uL pSK33 sample
    b. 5uL Cutsmart
    c. 0.5uL enzyme
    d. 35.5 uL H2O
    e. 37 C for ~1.5hr and 80 C for 20 min
- Gibsoned EBL1 and transformed DH5a (plus negative control without Gibson MM)
    a. Frag 1: 25 ng → 0.449 uL
    b. Frag 2: 10.34 ng → 0.179 uL
    c. Frag 3: 17.90 ng → 0.210 uL
    d. Frag 4: 17.17 ng → 0.191 uL
    e. Frag 5: 13.83 ng → 0.110 uL
    f. water up to 5uL
    g. 5uL Gibson MM
- Inoculated 20mL more of (+) colony
- Streaked (+) colony
- Streaked enterobacter

August 12th

AP

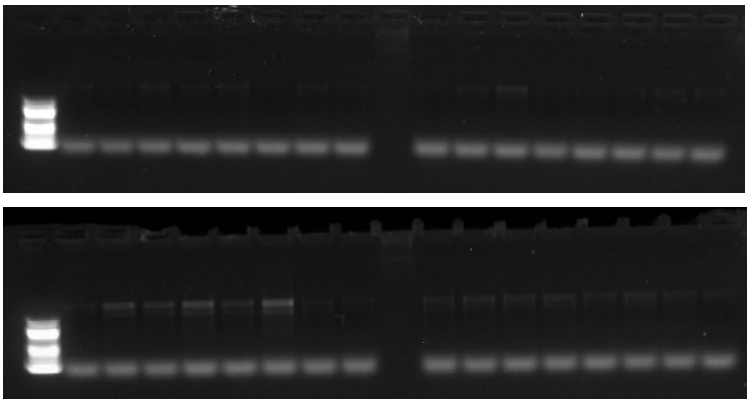
Optimizing Electroporation of Transposomes:

- Form transposomes (JL)
- Desalt transposomes using drop dialysis
- Transform:
    - Dh5 alpha + 1 µL transposomes (arctime 5.2)
    - Dh5 alpha + 2 µL transposomes (arctime 5.1)
    - Dh5 alpha + 1 µL desalted transposomes (arctime 5.1)
    - Dh5 alpha + 2 µL desalted transposomes (arctime 5.1)

- 2.5 kV, 2.0-mm cuvette

AG

- Saw growth on neg control plate still (less than before)
- CPCR of region 3 for 32 colonies
    - No positives



ladder/ cpcr region 3 colony 1/ 2/ 3/ 4/ 5/ 6/ 7/ 8/ 9/ 10/ 11/ 12/ 13/ 14/ 15/ 16
ladder/ 17/ 18/ 19/ 20/ 21/ 22/ 23/ 24/ 25/ 26/ 27/ 28/ 29/ 30/ 31/ 32

<u>Troubleshooting Ideas</u>

- Try PCR of pSK33 with 0.1ng and 0.5ng of plasmid template in a 50uL PCR rxn and up cycle number to 30
- Digest PCR product with DpnI restriction enzyme to destroy plasmid template
    - NEB protocol:
    - 8uL PCR rxn
    - 1uL DpnI
    - 1uL Cutsmart
    - Incubate at 37C for 30 min and heat inactivate at 80C for 20 min
- Extended Gibson incubation shown to help efficiency
- PCR amplify Gibson product (RCA)
- Assemble two smaller plasmids and combine them

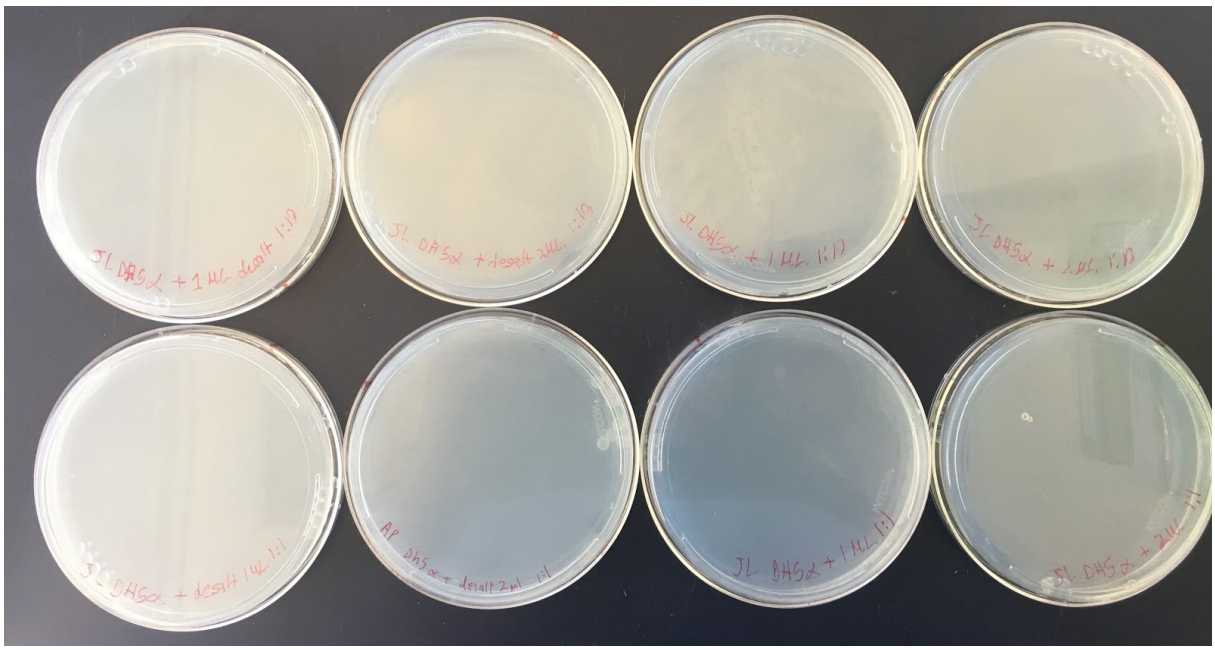
August 13th

AG/OE

- PCR of pSK33 with 0.1 ng and 0.5 ng of template instead of 1 ng
    - DpnI treated
    - gel purified

AP/ JL

16h at 37 C



-Desalted transposome 1 ul (arctime 5.10)
-Desalted transposome 2 ul (arctime 5.10)
-Transposome in regular buffer 1 ul (arctime 5.20)
-Transposome in regular buffer 2 ul (arctime 5.10)

No growth on all
Troubleshoot:

1. *Transposase concentration from Nate too low (Play around with 4ul in forming transposomes)*
2. *Transposomes/ transposon not forming properly→ assay for function by transforming transposed pUC19*
3. ~~Transposome not getting in (since 55kda per unit transposase)~~
4. ~~Transposome with multiple insertions in gDNA (kills it)~~

August 16th

AG

- inoculate positive colony to see if the toxin region is assembled

AP/ JL

- React transposase with pUC19, PCR purify
- Nanodrop concentration:
- Transform 1 ul with Dh5 alpha
    - 2.5 kV, arctime 4.8, 2.0-mm gap cuvette
    - Plate on kan 25

OE

- PCR amplify fragments together such that you have a new fragment consisting of the old fragments 1 and two. The same was done with fragments 4 and 5 and an additional fragment with 3, 4 and 5.
    - The idea is to try and PCR fuse as many fragments as possible to reduce the number of fragments in the gibson
    - Because fragment 3 is so much smaller than the other fragments, it may have to be added to the end of the fused fragment 4 and 5 if we cannot PCR amplify the three combined in the first place

---

August 17th

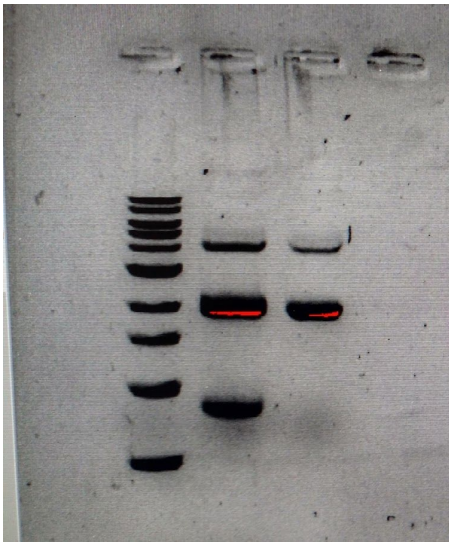
AG/OE

- Sent in pos colony for sequencing (primer on toxin region)
- Single cutter digestion of plasmid in pos colony (using NcoI-HF) to check for plasmid size
    - Expected: 11-12kb
    - Gel image saved on AGuo
- Fusion PCR step in protocol using already formed EBL2 frag 4 and DpnI treated pSK33 (made w/ 0.1 ng of template)
    - Ran 3 different reactions:

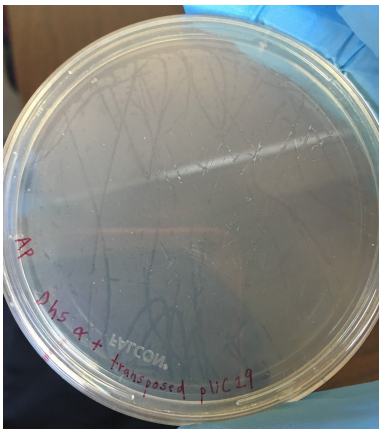
- Rxn 1:
  - 1ng F4, 0.7ng pSK33, 10.8uL H2O, 12.5uL Q5
- Rxn 2:
  - 2ng F4, 1.38ng pSK33, 9.12uL H2O, 12.5uL Q5
- Rxn 3:
  - 5ng F4, 3.45ng pSK33, 4.05uL H2O, 12.5uL Q5
- Ext time: 5min 28s
- Final ext 2 min
- 15 cycles
- Gel extract and PCR using Gibson primers (4F and MS 1R)

JL

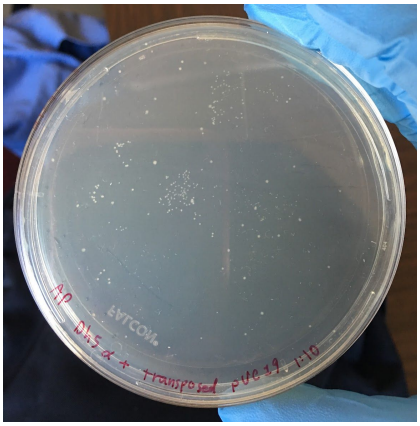
- React formed transposomes + pUC19
  1.5 µl EZ-Tn5 10X Reaction Buffer
  .33 µl target DNA (.2 µg)
  11.2 µl sterile water
  2 µl EZ-Tn5 Transposome
  15 µl Total reaction volume
- Transform that (assay if pre-formed transposomes are viable)



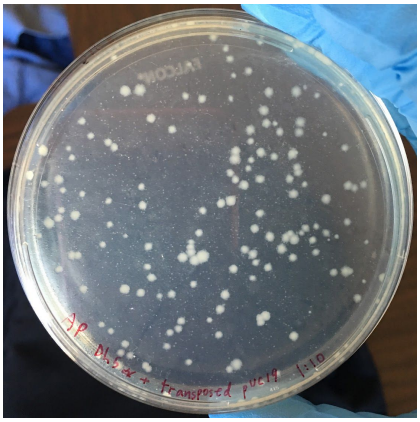
AP



Very few tiny colonies <kan25>



More colonies than 1:1 (???) <kan25>



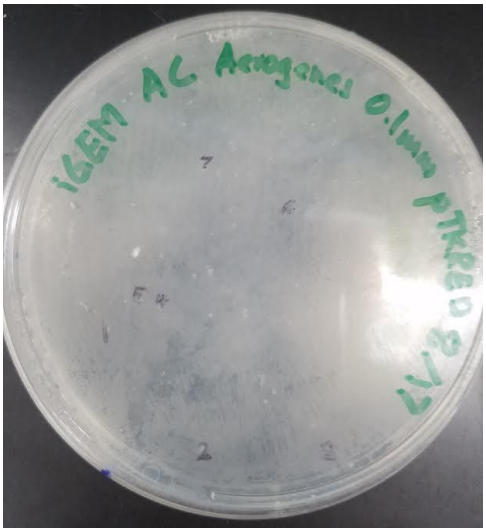
More colonies/Satellites than kan plates <amp 50>
Fungal Contamination → threw away amp plates

- Make amp plates
- Tomorrow: Re transform
  - Dh5 alpha + transposed pUC19 (amp 50) positive control
  - Dh5 alpha + transposed pUC19 (kan 25)

AC

- Prepared electro-competent aerogenes
  - Took overnight culture and added 0.5mL to 50mL LB (Did 2x of this)
    - Shake in 37C until reached OD of 0.463
  - Take both flasks out and place in ice water slurry

        - Swirl flasks in slurry for 5min
        - Let sit in slurry in the 4C cold room for 15min (All following work was performed at 4C or on ice)
    - Transfer culture to 4 falcon tubes (put 25mL of culture in each)
        - Spin tubes for 20min at 5000rcf
        - Remove supernatant
    - Resuspend each in 12.5mL Milli-Q Water at 4C
        - Mix suspensions so that 4x falcon tubes with 12.5mL each become 2x falcon tubes with 50mL each
    - Centrifuge at 5000rcf for 20min
        - Discard supernatant
        - Resuspend in Mili-Q water at 4C
        - Repeat this step once more
    - Resuspend pellet in 1mL water
        - Transfer into 1.5mL microcentrifuge tube
    - Centrifuge at 5000rcf for 20min
        - Discard supernatant
        - Resuspend using 30uL of Milli-Q water
- Electroporation
    - Add 2uL of pTKRED to 30uL of cells
        - Transform in 0.1cm cuvette
        - Arc time was 5.6
    - Add 2uL of pTKRED to 50uL of cells
        - Transform in 0.2cm cuvette
        - Arc time was 5.9
    - For both transformations
        - Immediately resuspend in 970uL of SOC
        - Incubate for 1hr at 30C
    - Centrifuge at 6000rpm on tabletop for 4min
        - Resuspend in 50uL of SOC
- Plating
    - Plate on Spect 25 plates
    - Use all of the 50uL SOC to plate
    - Incubate overnight at 30C



August 18th

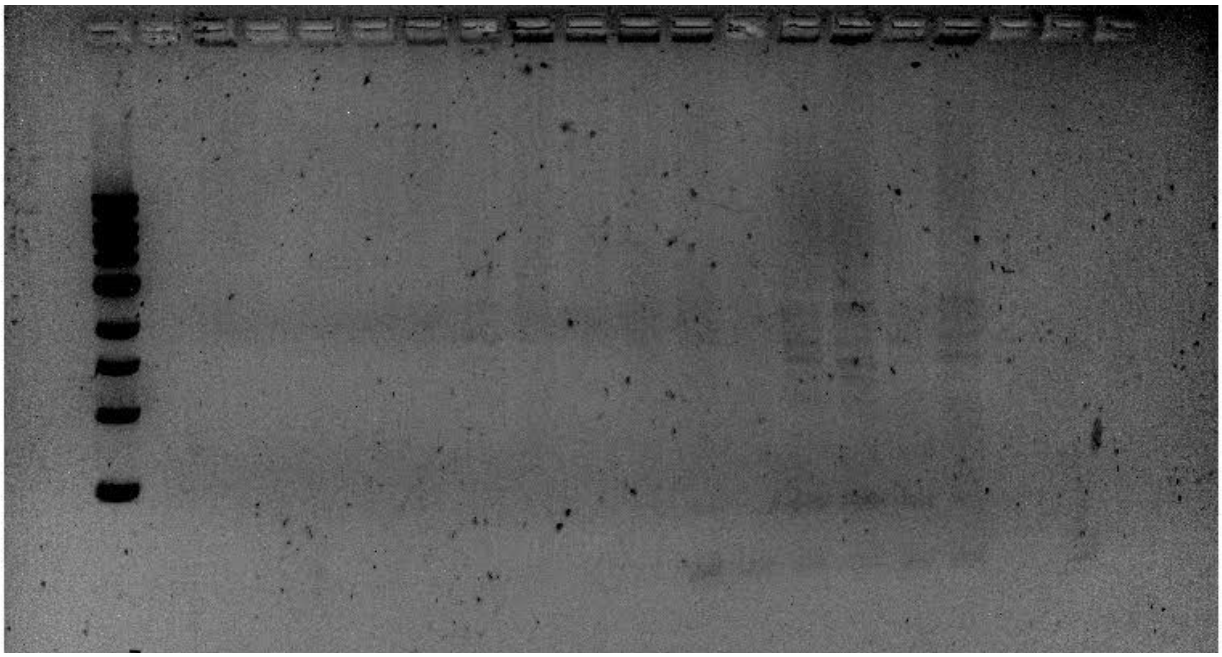
AP

- Re transform Dh5 alpha + transposed pUC19
- Arctime: 5.4, .2 cm cuvette
- 1:10 and 1:100 on kan25 and amp100

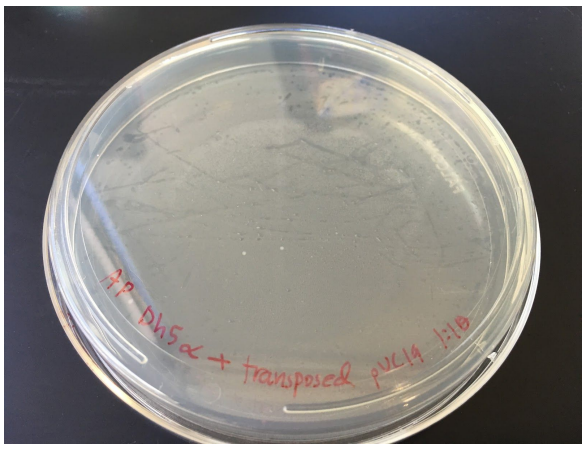
Results: 16h 20min at 37C

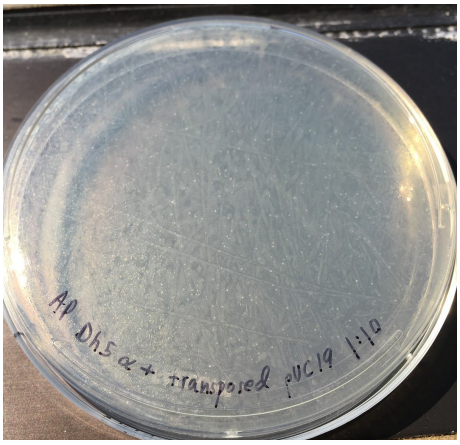
AC

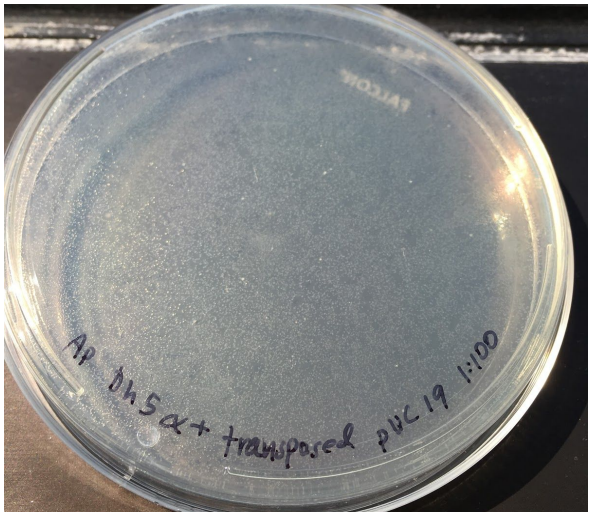
- Performed cPCR
    - Suspend 15 colonies in 30uL of dH2O
    - For 1 reaction:
        - 0.5uL of Forward Primer
        - 0.5uL of Reverse Primer
        - 12.5 Apex
        - 1uL colony suspension (one PCR tube was reserved for pTKRED plasmid stock to ensure PCR was not faulty)
        - Fill rest up to 25uL with dH2O
    - Cycling Conditions
        - 94C----->3min
        - 30x
            - 94C----->20s
            - 49C----->20s
            - 68C----->1min 10s
        - 68C----->5min
        - 14C----->Inifinite Hold
- Ran PCR products on 1% Agarose Gel with 1kb ladder and Sybrsafe

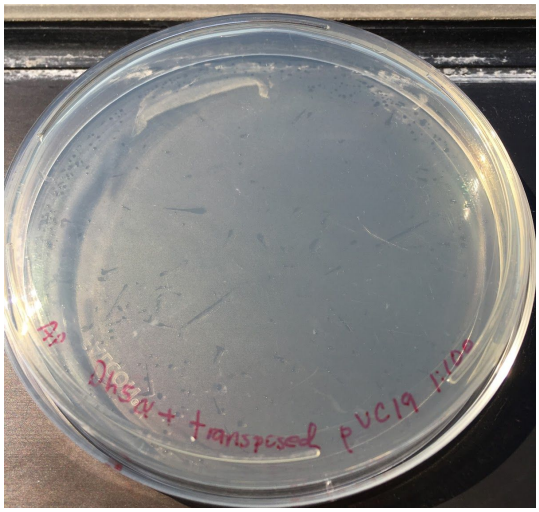


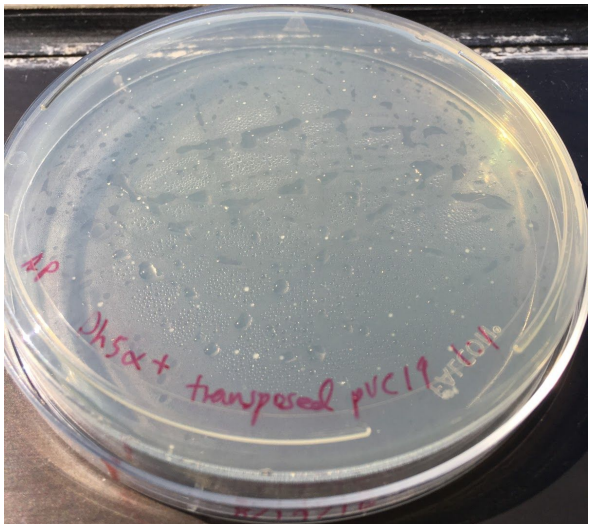
- Well 1 is ladder
- Well 2 is just pTKRED plasmid
- Wells 3-17 are colony PCRs
- Most likely PCR failed as not even the one with just the plasmid showed up
    - Will try different cycling conditions
    - Also do 50uL reaction as no bands showed up









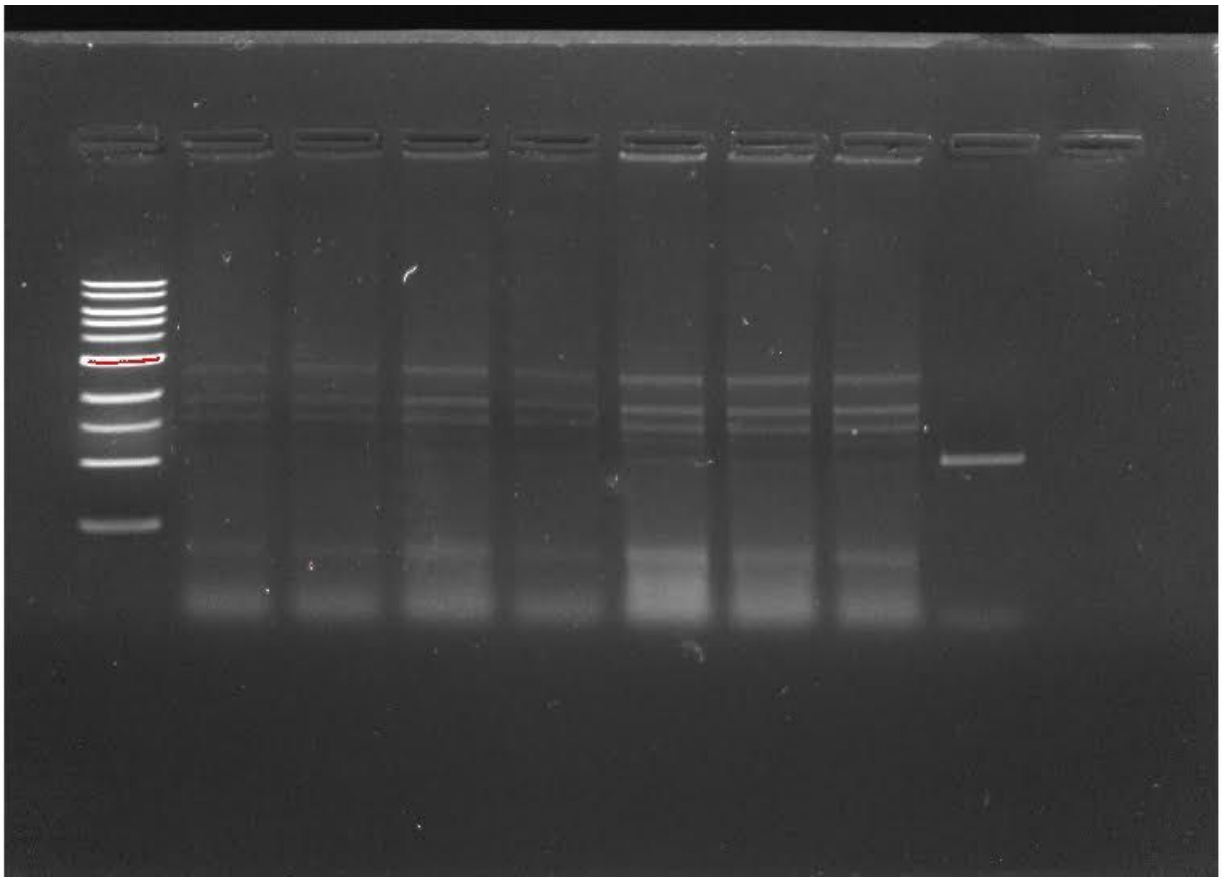


August 19th

AC

- Redid cPCR of pTKRED
    - Screened 7 colonies
    - For 1 reaction:
        - 25uL Apex
        - 1uL Forward Primer
        - 1uL Reverse Primer
        - 23uL Water
        - ½ of a colony added directly to PCR tube (Add 2ng pTKRED plasmid-stock to check PCR works and is fine-in a separate PCR tube)

- Cycling Conditions
  - 94C----->5min
  - 30x of
    - 94C----->30s
    - 49C----->30s
    - 68C----->1min 10s
  - 68C----->5min
  - 14C Infinite Hold
- Run PCR with 1kb ladder in 1% Agarose Gel with Sybrsafe

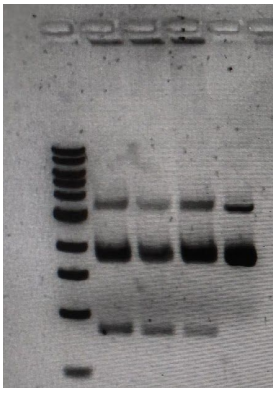


Well 9 is just pTKRED; Wells 2-8 are cPCR of colonies; 2nd ladder from bottom is 1kb

- Expected to see 1kb fragment same size as well 9
  - Lots of different bands showed up, most likely gDNA or other DNA

JL

Transposome activity assay (unsuccessful)



From left: ladder, normal reaction, 2X transposase, 3X transposase, pUC19 control

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August 20th

OE

- Ran a gel on the PCR conjoined fragments and nothing assembled correctly

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August 22nd

AC

- Inoculate 3 colonies overnight in 5mL LB with 2.5uL of SpecT antibiotic

OE

- Ran a gel for the the gibson a

August 23rd

AG

- Sequencing results back → exonuclease chewed back the strand enough s.t. there was compatible region between 3' end of pSK33 and 5' end of toxin region → ~140 bp deletion on toxin region (3 fragment plasmid made)
    - Order 5 primers to gradually add back toxin sequence (use KAPA)
        - try doing 5 cycles matching annealing temp of actual primer and 25 cycles matching annealing temp of primer + overhang if the product doesn't form with the long primer

---

August 26th

AG

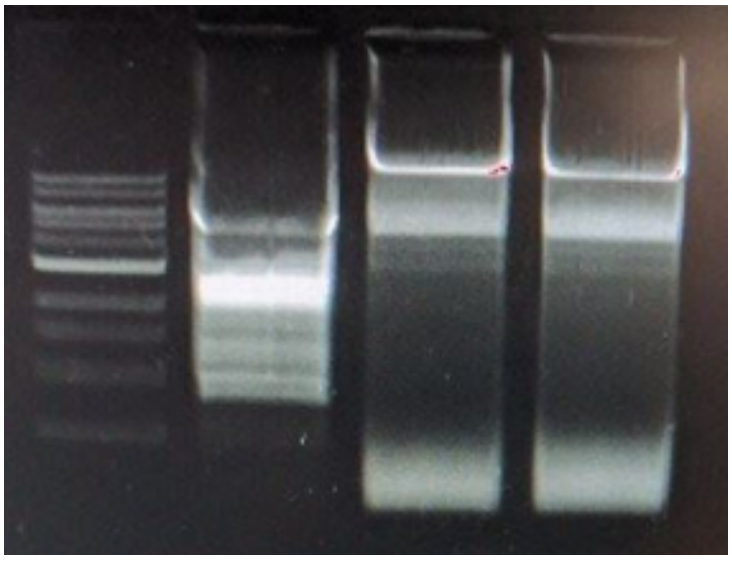
- PCR using psk33_fix_1R and EBL1_toxin_fix_1F
    - 12kb fragment successfully amplified using KAPA

- Gibsoned 11kb frag
    - CPCR region 3 amplify
        - 1uL of gibson
        - 0.5uL F
        - 0.5uL R
        - 12.5 Taq 2X MM
        - 10.5uL dH2O
        - Saw faint band at the right size

cpcr region 3/ 100bp ladder

- PCR fragment using gibson as template
  - First five cycles at 51C Tm and 20 cycles at 64C
  - No band at 11kb... probably bad primers
    - ordered new forward primer

- PCR using psk33_fix_1R and EBL1_toxin_fix_2F
  - Did one normal KAPA at Tm = 51 C
  - Did one with the first 5 cycles at 51 and 20 cycles at 65
    - worked better



ladder/ 11kb fragment/ normal KAPA protocol/ increased Tm protocol

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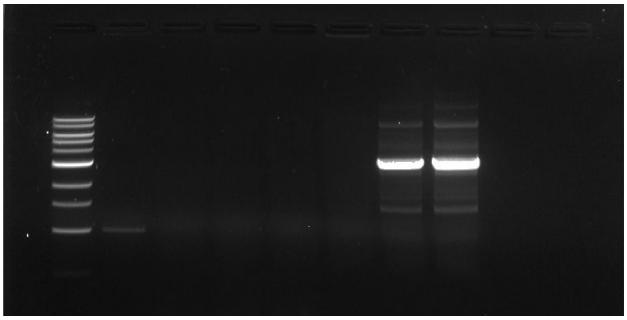
August 29th

AC

- Ran gel with EBL2 gDNA primers to verify Aerogenes

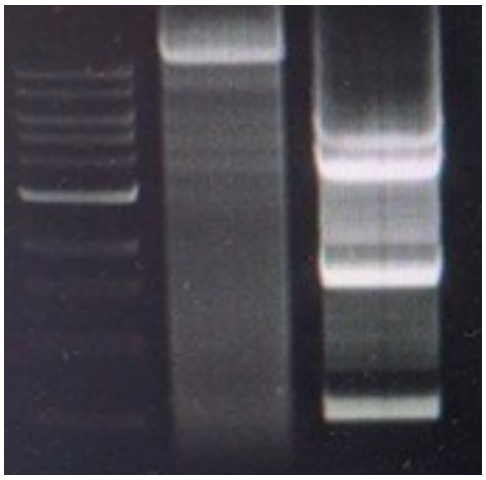


Wells

#1: 1kb ladder
#2-6: AG wells
#7-9: gDNA extraction from 3 different colonies
Only 2 colonies were Aerogenes

AG

- PCR using psk33_fix_1R and EBL1_toxin_fix_3F
- PCR using new 11kb frag primers

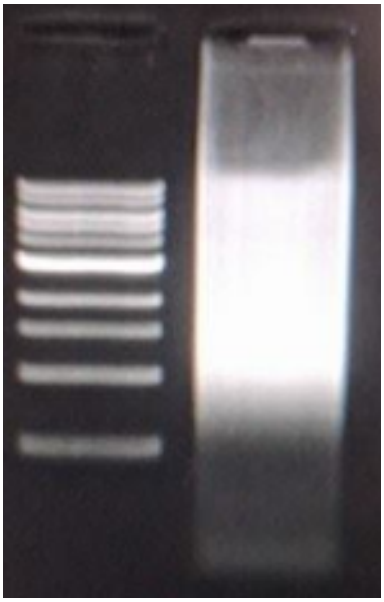


1kb ladder/ toxin fix/ 11kb frag

August 30th

AG

- Inoculate 15 colonies from devices 1-3 and pos/neg control in 5mL LB and 5uL cat for interlab study
- EBL1 toxin fix 1R + 4F didn't work → saw large smear instead of band
    - troubleshoot tmr

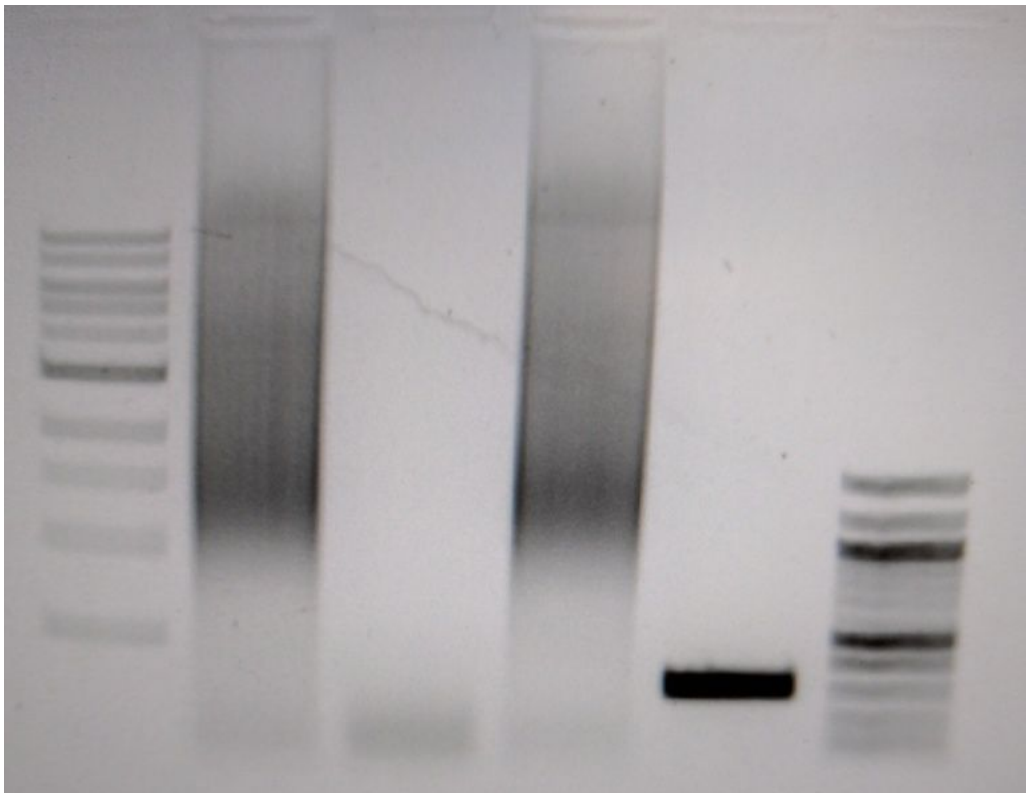


August 31st

AG

- Troubleshoot PCR w/ EBL1_toxin_fix_1R and EBL1_toxin_fix_4F
    - Use new dH2O
    - Try reaction mix without template DNA (check for carryover contamination)
    - Try going back down to 25 cycles (did 28 cycles last time) to check for overcycling

- Try 5 cycles annealing temp of annealing region and 20 cycles annealing temp of entire primer including overhang (54C → 68C) to increase specificity
- Try running PCR using CPCR region 1 primers and OneTaq with template DNA to verify correct sequence is in there
    - Results:
    - Gel image under AGuo (1kb ladder; 25 cycles 54C; no template control; 25 cycles 54 → 68 C; CPCR region 1; 100bp ladder)
    - Two smears for 25 cycles 54 C and 25 cycles 54 → 68C, faint band at correct size observed for both
    - CPCR region 1 positive → template is fine
    - No template control has only primer dimers → no contamination
    - **4F primer is the problem**



1kb ladder/ 25 cycles 54C/ no template/ 5 cycles 54 → 20 cycles 68C/ CPCR primers with template/ 100bp ladder